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FINAL TECHNICAL REPORT

# EXPERIMENTAL STUDIES FOR THE DETECTION OF PROTEIN IN TRACE AMOUNTS



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## ABSTRACT

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A method of detecting biological macromolecules, based upon the observation of spectral changes due to aggregation of a dibenzo-thiacarbocyanine dye when it is adsorbed on the macromolecular structure, was investigated. The effects of pH, temperature, and electromagnetic radiation on the stability of the dye were first determined. The dye is stable over the pH range 3.8 to 9.6 and unaffected by storage at temperatures below 60°C, but it is unstable when exposed to intense light. The aggregation state of the dye in solution is altered by solvent composition, dye concentration and temperature, but not by pH.

The reactions of the dye with inorganic salts, microorganisms, pollen, polypeptides, simple proteins, conjugated proteins, synthetic polypeptides, polynucleotides, carbohydrates, amino acids, pyrimidine and purine bases, nucleosides and nucleotides were investigated. In trace amounts (less than 0.002 percent), only proteins, synthetic polypeptides, nucleic acids, microorganisms, pollen and substituted polysaccharides cause changes in the absorption spectrum of the dye. Mono-, di-, and trisaccharides, purine and pyrimidine bases, amino acids and nucleosides have no effect. Polypeptides and nucleotides are usually effective only at higher concentrations; the action of the inorganic salts depends principally upon the nature of the anion. The influence of such variables as pH, temperature and dye-macromolecule stoichiometry on the stability and formation of the dye-macromolecule complexes and dye aggregates were also determined. The optimum conditions for dye-macromolecule formation and stability appear to be a pH of 7-9, dye concentration of $4 \times 10^{-5}$M, and a temperature of 20-40°C. The relationship of macromolecule structure to the absorption spectrum of the macromolecule-dye complex was examined; it was found, especially in the case of DNA, that the nature of the absorption spectrum of the dye-macromolecule complex could give significant information about the structure of the macromolecule.

The macromolecule-dye reaction was investigated for its applicability to detection of macromolecules in heterogeneous materials by observing its behavior with soil extracts and suspensions of microorganisms. The results are especially encouraging for the possible application of the dye reaction to the detection of macromolecules on extraterrestrial bodies.

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# SECTION 1

# INTRODUCTION

The color changes which occur when certain dyes stain tissue elements (metachromasia) have long served to identify biological materials and to enhance their visibility in tissue preparations. A recent review by Schubert and Hamerman[1] has extensively covered the chemical theory and histochemical use of metachromasia. In general these color changes are due to aggregation of the dye molecules when adsorbed on the biological material. The aggregation produces several new molecular species with absorption spectra having peaks at shorter wave lengths than the absorption spectrum of the simple dye molecules. Likewise, the interaction of acridine dyes with polyanions in solution produce spectral changes which have been explained as a function of the aggregation of the dye bound to the polyanion[2-6]. In this study we have been especially concerned with the interaction of cyanine dyes with biological material. The cyanine dyes are important sensitizing dyes which have found wide use in the photographic process[7] and consequently, their behavior in solution and as films has been studied extensively. When these dyes are prepared in aqueous solutions, increasing their concentration produces an aggregation of the dye molecules, and most cyanine dyes exhibit new bands at shorter wave lengths than the mono-molecular spectrum. However, some of these dyes which have a planar structure, on further increase in concentration of the aqueous solution, develop an intense, sharp absorption maximum at longer wave length than the molecular absorption. This effect has been observed by a number of investigators[8-15] and the new intense absorption band is often referred to as a J-band. The J-bands appear to result from interaction of large numbers of molecules in an orderly array and Jelly[15] concludes that the dye molecules are arranged parallel to each other. Sheppard[16] suggests that water molecules linking the polar groups of the dyes at the edges of pairs of molecules assist in formation of the aggregate state. Of especial significance to the present

study are the observations by Sheppard[8] and Natanson[9] that traces of protein favor aggregation of certain carbocyanine dyes to give a J-band. In view of the previously cited work on the interaction of acridine orange and polyanions and the cationic nature of the carbocyanine dyes it seems reasonable to conclude that the dye aggregates in the presence of protein by adsorption of the dye molecules onto the protein. If this is the case, there is reason to believe that the nature of the protein, or for that matter other polymers, influences the degree of aggregation and therefore the type of spectral changes which occur when the dye and the polymer interact. Furthermore, the extremely small amounts of protein required for interaction with the dye molecules suggests that the spectral changes which occur may be used to detect trace amounts of macromolecules.

The principal objectives of this study are to:

(a) Observe the spectral changes resulting from interaction of various macromolecules with a carbocyanine dye which was selected for this work (3,3'diethyl 4,5-4'5'dibenzo-9-methyl thiacarbocyanine bromide),

(b) Study some of the factors in regard to structure of the macromolecules which influence the nature of the spectral changes, and

(c) Examine the feasibility of using the observed changes as criteria for the detection and characterization of macromolecules.

This report summarizes the results which have been obtained during this investigation.

## SECTION 2

## METHODS

Where microorganisms were used in experiments, these were cultured and prepared in our laboratory; all other test materials were obtained commercially. Two samples of the dye 3,3'diethyl-9-methyl 4,5,4'5' dibenzothiacarbocyanine bromide were gifts of F. W. Mueller, Ansco, and J. A. Leermakers, Eastman Kodak Company. Absorption spectra were determined from 400 to 700 mμ with the use of either a Model 14 Cary recording spectrophotometer or a Beckman DK-2A recording spectrophotometer. Absorption cells having path lengths of 0.1 to 10 cm were employed and the reference solution used is indicated in each experiment. Dye and test materials were prepared from weighed amounts of the materials, except as otherwise indicated.

# SECTION 3

# PROCEDURE AND RESULTS

## 3.1 SELECTION OF THE EXPERIMENTAL DYE

The carbocyanine dyes listed in Table I were prepared as aqueous solutions and mixed with equal volumes of water, 0.02 percent deoxyribonucleic acid (DNA), or 0.002 percent gelatine solutions. The absorption spectra of the DNA and gelatine solutions were determined and compared with that of the aqueous dye solution. The absorption spectra of dyes 3, 4, 6, 7 and 8 were altered by the presence of DNA. On the other hand, only dyes 6, 7 and 8, all of which are substituted at the 5,5' and/or the 4,4' position, were affected by the addition of gelatine, and all of these dyes formed J-bands at about 650 $m\mu$. Dye number eight formed the most intense J-band with gelatine and it also exhibited the greatest changes in the presence of DNA. Since dye number eight appeared to interact most favorably with both DNA and gelatine, it was selected as the test dye for use in subsequent experiments. Its structural formula is shown in Figure 1.

## 3.2 THE EFFECTS OF ENVIRONMENT ON THE ABSORPTION SPECTRUM OF THE DYE

In order to properly assess the influence of added materials on the absorption spectrum of the dye, it is necessary to know how the environment affects the dye in solution. There are two principal parameters by which the effects of environmental changes on the dye solution can be evaluated: absorbance, and the wave length at which maximum absorption takes place. We have used changes in absorbance to estimate the chemical stability of the dye and have ascertained the effects of electromagnetic radiation, temperature and changes in pH on this parameter. On the other hand, changes in the position of the absorption maximum have been used as criteria for alterations in the degree of aggregation of the dye.

TABLE I

CARBOCYANINE DYES TESTED FOR THEIR INTERACTION WITH MACROMOLECULES

(Dilute solutions of each dye were mixed with equal volumes of 0.020 percent DNA, 0.002 percent gelatin, or water, and the absorption spectra of the solutions determined in a 1 cm cell against a water blank.)

| No. | Dye | Concentration of Dye M | Absorption Maxima of Aqueous Dye Solution Plus | | |
|---|---|---|---|---|---|
| | | | Water mμ | 0.01% DNA mμ | 0.001% Gelatin mμ |
| 1. | 3,3' diethyl-9-ethyl selenocarbocyanine iodide | $1 \times 10^{-4}$ | 555 | 555 | 555 |
| 2. | 3,3' diethyl-9-ethyl thiacarbocyanine iodide | $4 \times 10^{-5}$ | 545<br>505 | 545<br>505 | 545<br>505 |
| 3. | 3,3' diethyl-9-ethyl oxacarbocyanine iodide | $1 \times 10^{-4}$ | 475 | 468<br>490 | 475 |
| 4. | 3,3' diethyl-9-methyl thiacarbocyanine iodide | $4 \times 10^{-5}$ | 503<br>540 | 510<br>545 | 503<br>540 |
| 5. | 3,3' diethyl-9-methyl oxacarbocyanine iodide | $1 \times 10^{-4}$ | 467 | 467 | 467 |
| 6. | 3,3' dimethyl-5,5'-dichloro-9-ethyl thiacarbocyanine chloride | $4 \times 10^{-5}$ | 510<br>550 | 555 | 510<br>550<br>650* |
| 7. | 3,3' dimethyl, 4,5-4'5' dibenzo-9-ethyl thiacarbocyanine chloride | $4 \times 10^{-5}$ | 506 | 575 | 510<br>656 |
| 8. | 3,3' diethyl, 4,5-4'5' dibenzo-9-methyl thiacarbocyanine bromide | $4 \times 10^{-5}$ | 510 | 575**<br>535*** | 510<br>650 |

* Concentration of gelatin must exceed 0.001 percent to induce peak at 650 mμ.

** Native DNA

*** Denatured DNA

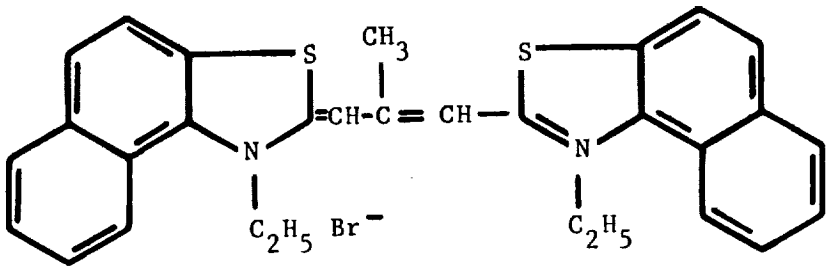


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FIGURE 1. STRUCTURAL FORMULA OF DYE NUMBER 8, 3,3' DIETHYL, 4,5,4',5' DIBENZO-9-METHYL THIOCARBOCYANINE BROMIDE.

### 3.2.1 EFFECT OF ELECTROMAGNETIC RADIATION, pH, AND TEMPERATURE ON THE ABSORBANCE OF THE DYE SOLUTION

Solutions of the dye when exposed to high light intensities quickly bleach from a deep fuchsia color to a light yellow color. On the other hand, exposure to 10,000 rad of x-irradiation had no effect on the absorbance of the dye solution (Figure 2). The aqueous dye solution exhibited some decrease in absorbance during the first 24 hours at all temperatures tested. However, the changes were not large unless the temperature was greater than 40°C and it appears that the initial decreases at lower temperatures may be due to adsorption of the dye on the containers, since small amounts of adsorbed dye were found on the glass at the end of the experiment and storage at 22°C for an additional 37 days did not cause a further decline in absorbance (Figure 3).

The absorbance of the dye solution was not appreciably altered by changes in pH over the range 3.8 to 9.6, but at a pH of less than 3.8 or more than 9.6, absorbance decreased rapidly (Figure 4). However, it should be noted that when the dye is adsorbed on a macromolecule it is much more stable to pH changes and, in fact, does not appear to be affected by pH unless it is desorbed (see Sections 3.4.1 and 3.5.1). Because of the unfavorable influence of pH and light on the free dye in solution, subsequent experiments were carried out where possible in pH 6.9-7.0, 0.017 M cacodylic acid buffer, or pH 8.8, 0.001 M tris buffer, and in containers wrapped in aluminum foil.

### 3.2.2 EFFECT OF pH, SOLVENT, DYE CONCENTRATION, TEMPERATURE, AND INORGANIC IONS ON THE WAVE LENGTH OF THE DYE ABSORPTION MAXIMUM

The influence of environment on the wave length of the absorption maximum is, for our purposes, a most important factor. It has previously been shown that thiacarbocyanine dyes will aggregate in solution and that the degree of aggregation is indicated by the nature of the shift in the absorption maximum[8]. This type of behavior is, of course, not limited to the thiacarbocyanine dyes, but is seen in numerous other dyes[1]. It appears that in organic solvents and at low concentrations in water, dyes are in a monomeric state characterized by a definite absorption maximum. Aqueous solutions of higher concentrations give new absorption maxima in the shorter wave length region. In the case of the thiacarbocyanine dyes, the presence of electrolytes or still higher concentrations of the dye results in another new absorption band which, with respect to the maximum corresponding to the monomeric state of the dye, is displaced towards the long wave length region of the spectrum. These new absorption maxima correspond to polymeric forms of the dye; the appearance of the short wave length maxima are indicative of the formation of dimers and trimers, and the long wave length

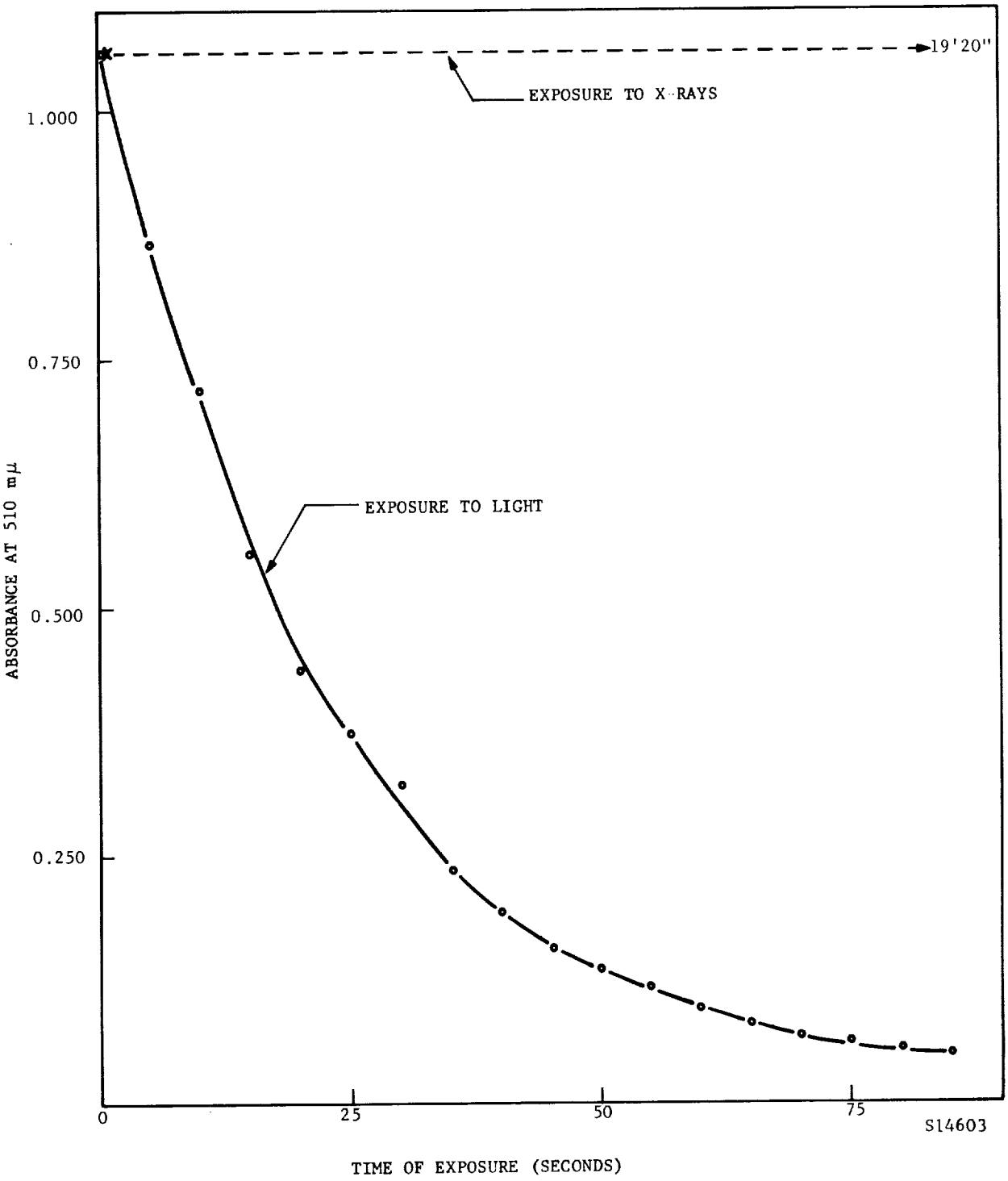


FIGURE 2. EFFECT OF ELECTROMAGNETIC RADIATION ON THE ABSORBANCE OF THE AQUEOUS DYE SOLUTION

A 2 x $10^{-5}$M dye solution was exposed in 1.0 cm cells to radiation, filtered through 4 1/2 inches of water, from a 375W incandescent lamp or to 10,000 rad of 150 KV(0.22 mm Cu hvl 0.1 mm Cu plus 1.0 mm Al filter) x-rays. At the times indicated in the figure, samples of the dye were taken and the absorbance at 510 mμ determined. Absorption measurements were made in a 1.0 cm cell against a water blank.

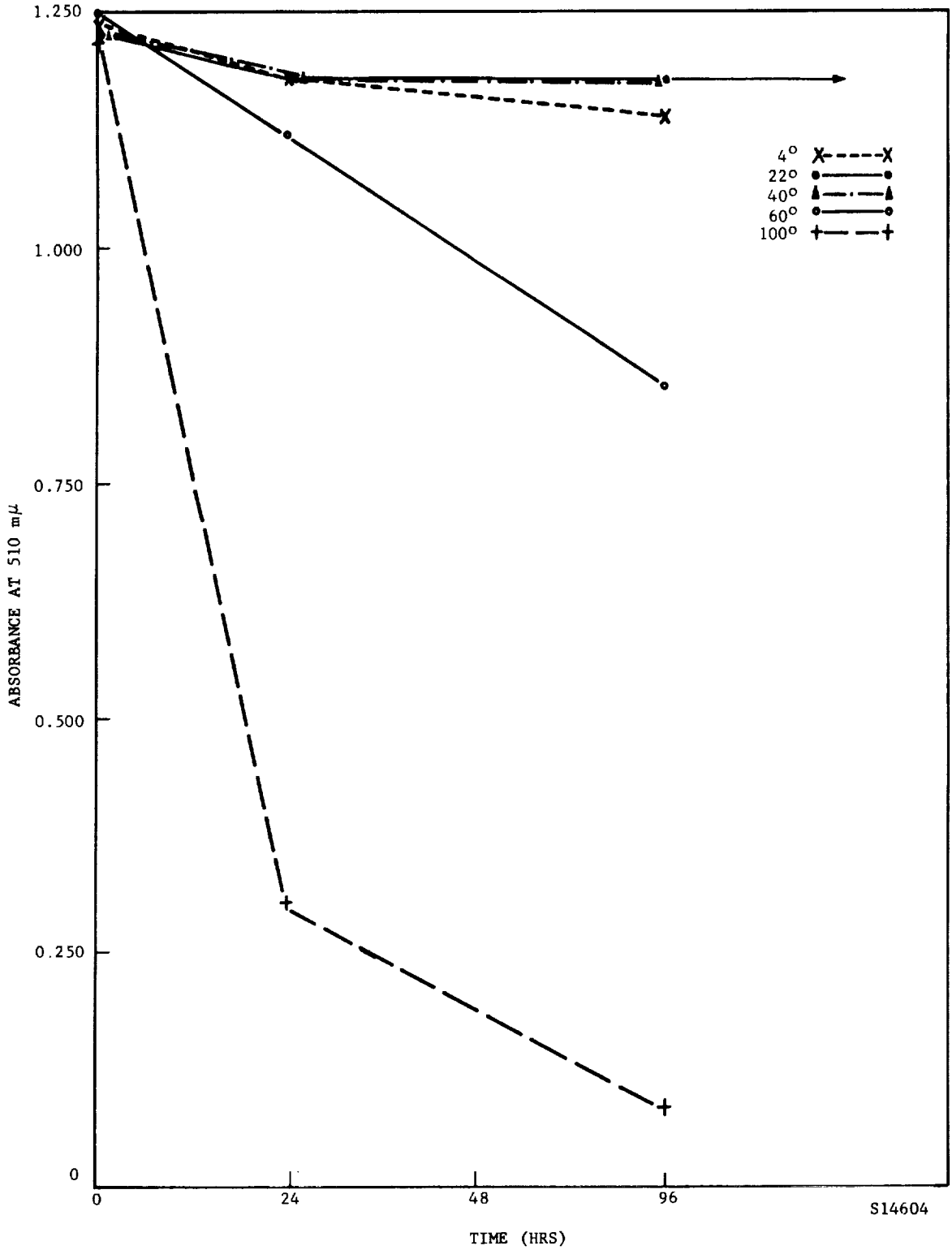


FIGURE 3. EFFECT OF TIME AND TEMPERATURE ON THE ABSORPTION OF THE AQUEOUS DYE SOLUTION.

$2 \times 10^{-5}$M dye solutions were maintained at constant temperatures. At the times indicated in the figure, a sample of each solution was brought to room temperature and the absorbance determined. Measurements were made in a 1.0 cm cell against a water blank.

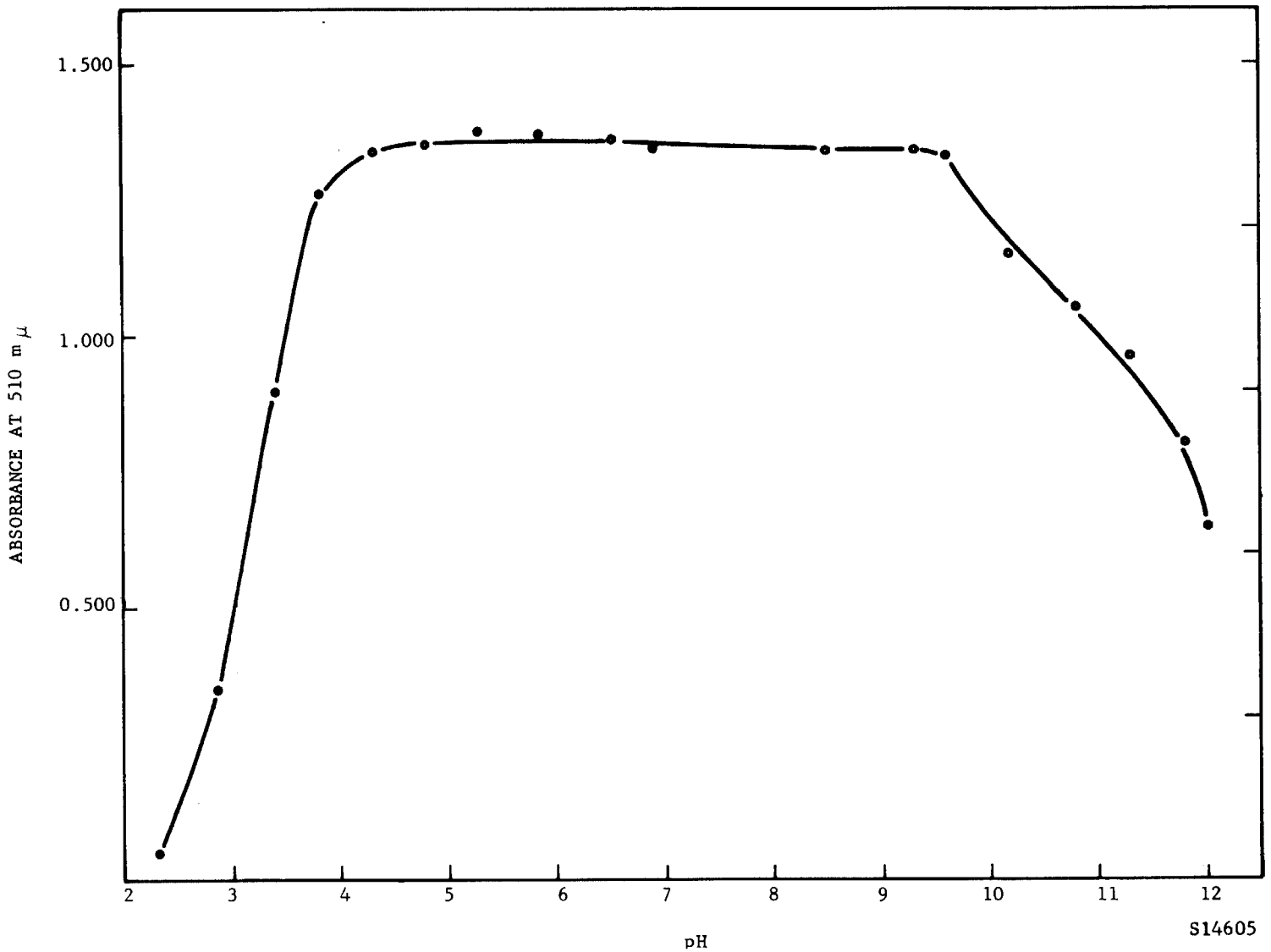


FIGURE 4. EFFECT OF pH ON THE ABSORBANCE OF THE AQUEOUS DYE SOLUTION.

Two aliquots of a $2 \times 10^{-5}$ M dye solution were used. The pH of one sample was decreased by the addition of 1N HCl and the pH of the second aliquot was increased by the addition of 1N NaOH. Changes in volume due to the additions of HCl or NaOH were negligible. At each pH indicated in the figure, the absorption spectrum of the dye solution was determined and the absorbance at 150 mμ ascertained. Measurements were made in a 1.0 cm cell against a water blank.

maximum (J-band) corresponds to a higher state of aggregation. Thus, by altering the concentration of the dye, the solvent composition, or the electrolyte content of the solution, it should be possible to obtain a number of absorption maxima and relate them to the relative degree of dye aggregation. Once these maxima have been determined, they can be compared with the wave lengths of the maxima formed by adsorption of the dye on macromolecules. By analysis of the absorption spectrum of the dye-macromolecule complex some information may then be obtained which will relate the nature of the macromolecule to the degree of dye aggregation which takes place when the dye is adsorbed.

Changes in pH over the range 2.3 to 12 had no effect on the position of the absorption maximum of an aqueous $2 \times 10^{-5}$M dye solution.

The absorption spectra of the dye dissolved in water and ethanol are shown in Figure 5. The two spectra are quite different. The absorption maximum is at 575 mμ in ethanol and 510 mμ in water, and the intensity of the absorption peak is 57 percent greater in the ethanol solution than in the water solution. When the dye is dissolved in a series of solutions in which the ethanol content is continually dimished, the absorption spectrum does not undergo a change until the ethanol content is 50 percent or less (Table II). However, as the ethanol content is brought below this level the wave length of the maximum shifts towards the shorter end of the spectrum and the absorbance at the maximum diminishes. At an ethanol content of 30 percent, two distinct maxima appear, one at 572 mμ and the other at 533 mμ (Figure 5). As the ethanol content is decreased further, the maxima appear at slightly shorter wave lengths and the absorbance at the longer wave length maximum decreases, while the absorbance at the shorter wave length maximum increases. However, when the alcoholic content drops to 10 percent, the longer wave length maximum is no longer apparent and the remaining maximum is less intense and appears at still shorter wave lengths of 525-510 mμ.

In aqueous solution, the position of the dye absorption peak varied with the concentration of the dye. The wave length of the absorption maximum continually increased as the concentration of the dye was decreased over the range $1.2 \times 10^{-4}$ to $5 \times 10^{-7}$M and in very dilute solutions two absorption maxima were evident; one at about 530 mμ and the other at 570 mμ (Table III). With decreasing dye concentration there was initially a very gradual shift in the position of the absorption maximum from 500 to 510 mμ, but at a dye concentration of $5 \times 10^{-6}$M a more abrupt shift to the 530 mμ region of the spectrum occurred, and at a concentration of $1 \times 10^{-6}$M a second peak was evident at 570 mμ. Alterations in temperature likewise produced changes in the position of the absorption maximum. With an increase in temperature there was a gradual shift in the position of the

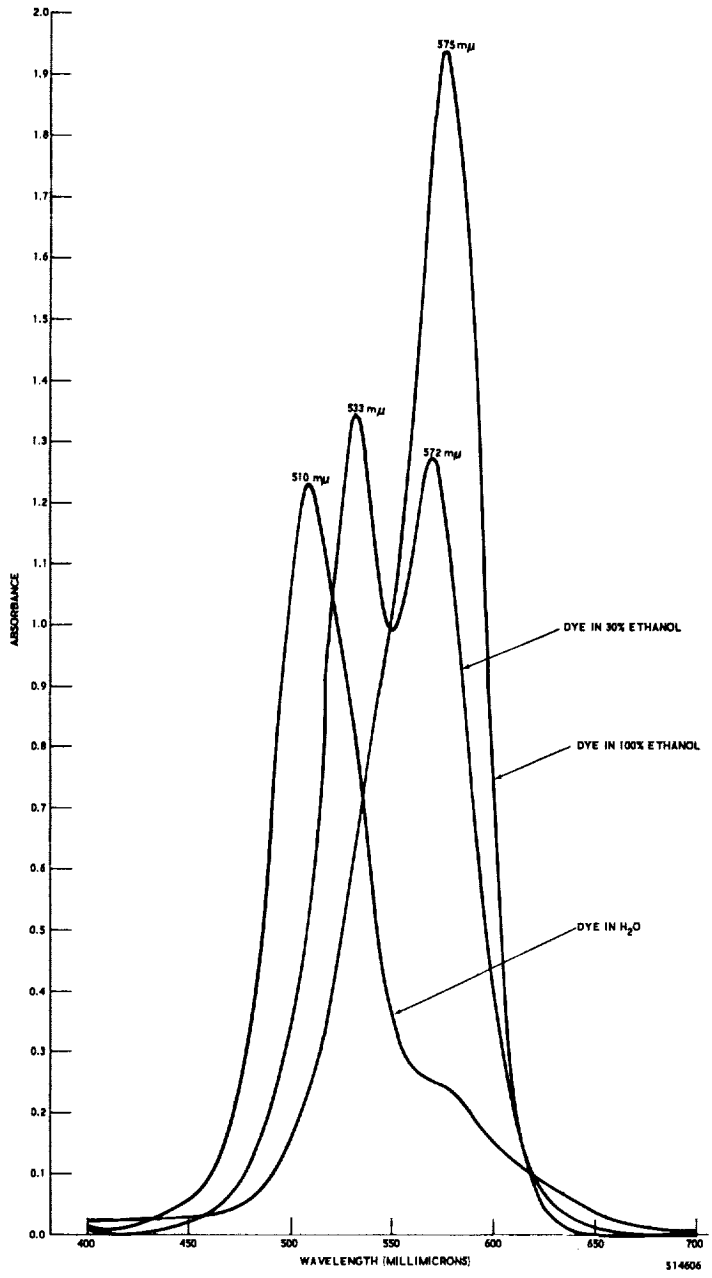


FIGURE 5. EFFECT OF SOLVENT COMPOSITION ON THE ABSORPTION SPECTRUM OF THE DYE.

In each case, the dye concentration was 2 x $10^{-5}$M and the measurements were made using a 1.0 cm cell and a water blank.

TABLE II

EFFECT OF SOLVENT COMPOSITION ON THE ABSORPTION MAXIMUM OF THE DYE IN ETHANOL-WATER SOLUTION

($2 \times 10^{-5}$M dye solutions having the indicated ethanol content were prepared by diluting a concentrated ethanol solution of the dye with water. The absorption spectrum of each solution was determined using 1.0 cm cells and water as a blank. The positions and intensities of the absorption maxima were obtained by inspection of the absorption spectra.)

| Percent Ethanol | Wave Length of Absorption Maxima ($m\mu$) | Absorbance at Maximum |
|---|---|---|
| 100 | 575 | 1.930 |
| 90 | 575 | 1.970 |
| 80 | 575 | 1.970 |
| 70 | 575 | 1.970 |
| 60 | 575 | 1.970 |
| 50 | 573 | 1.906 |
| 40 | 572 | 1.760 |
| 30 | 572, 533 | 1.270, 1.340 |
| 20 | 570, 530 | 0.720, 1.490 |
| 10 | 525, 515 | 1.210, 1.215 |
| 0 | 510 | 1.230 |

TABLE III

EFFECT OF DYE CONCENTRATION ON THE POSITION
OF THE ABSORPTION MAXIMUM IN AN AQUEOUS DYE SOLUTION

(Aqueous solutions having the indicated dye concentrations were prepared by diluting a $1.2 \times 10^{-4}$M solution of the dye. The absorption spectra of these solutions were determined at 25°C and the wave length of the absorption maximum obtained by inspection of the absorption curves. Measurements were made against water in cells having path lengths of 0.1 to 10 cm. All solutions were buffered at pH 7.0 with 0.017M cacodylic acid buffer.)

| Dye Concentration (M) | Absorption Maxima (mμ) |
|---|---|
| $1.2 \times 10^{-4}$ | 500 |
| $8 \times 10^{-5}$ | 502 |
| $6 \times 10^{-5}$ | 505 |
| $4 \times 10^{-5}$ | 506 |
| $2 \times 10^{-5}$ | 509 |
| $1 \times 10^{-5}$ | 510 |
| $5 \times 10^{-6}$ | 527 |
| $1 \times 10^{-6}$ | 528 and 570 |
| $5 \times 10^{-7}$ | 530 and 570 |

absorption maximum from 510 mμ at 11°C to 515 mμ at 43°C. At 51°C two peaks appeared, one at 520 mμ and the other at 530 mμ. With an increase in temperature to 69°C, the 520 mμ peak disappeared, the 530 mμ maximum became more intense and a new peak was found at 575 mμ. Increasing the temperature still higher caused an increase in absorbance at 575 mμ and a decrease in the intensity of the 533 mμ maximum (Table IV).

It is well known that the addition of electrolytes to cyanine dye solutions will cause the dye molecules to aggregate[7,8,11,15]. This phenomenon is illustrated in Figure 6 which shows the absorption spectrum of a dye solution to which $MgCl_2$ has been added. Compared to the aqueous dye solution, the absorption spectrum has been greatly altered. A very intense, narrow absorption maximum (J-band) appears at 650 mμ, a second maximum occurs at 555 mμ, and prominent shoulders are seen at 512 mμ and 450 mμ.

Decreases in dye concentration, increases in temperature, and increases in the ethanol content of the solvent systems are all factors which tend to increase the dispersion of dye molecules, whereas the addition of electrolyte usually promotes aggregation. In each case, when the factor investigated was altered so as to increase dispersion, the absorption maximum was shifted to longer wave lengths. As the dispersive effect was strengthened, definite absorption maxima appeared at the following wave lengths and in the sequence indicated: 510, 533 and 575 mμ. On the other hand, the effect of adding electrolyte was quite different. New absorption peaks were found at both longer and shorter wave lengths than that exhibited by the aqueous dye solution. These new peaks are at 650, 555 and 450 mμ.

From this information, it appears that at least six distinct dye species, representing different states of dye aggregation, can be formed. Arrangement of the maxima, which are taken as indicative of each molecular species, in order of increasing degree of dye aggregation results in the following sequence: 575, 555, 533, 510, 450 and 650 mμ. The aggregate represented by the 650 mμ maximum is evidently relatively large since it is sedimented by centrifugation, whereas the other aggregates are not.

## 3.3 INTERACTION OF THE DYE WITH VARIOUS SUBSTANCES

### 3.3.1 INORGANIC SALTS

In aqueous solutions at pH 7.0, the dye is cationic in nature and is present in a somewhat aggregated state as evidenced by the information obtained in the previous experiments and the shift in the absorption

TABLE IV

EFFECT OF TEMPERATURE ON THE POSITION OF THE ABSORPTION MAXIMUM IN AN AQUEOUS DYE SOLUTION

(The absorption spectra of a $2 \times 10^{-5}$M solution of the dye in pH 7.0, 0.017M cacodylic acid buffer were determined at the indicated temperatures and the wave lengths of the absorption maxima were obtained by inspection of the curves obtained. Measurements were made against water using cells with a path length of 1.0 cm.)

| Temperature (°C) | Absorption Maxima (mμ) | Absorbance at Absorption Maximum |
|---|---|---|
| 11 | 501 | 1.224 |
| 19 | 505 | 1.178 |
| 23 | 506 | 1.117 |
| 29 | 510 | 0.980 |
| 34 | 513 | 0.930 |
| 43 | 515 | 0.900 |
| 51 | 520, 530 | 0.859, 0.849 |
| 69 | 575, 530 | 0.432, 0.900 |
| 79 | 575, 533 | 0.530, 0.850 |
| 96 | 575, 533 | 0.672, 0.701 |

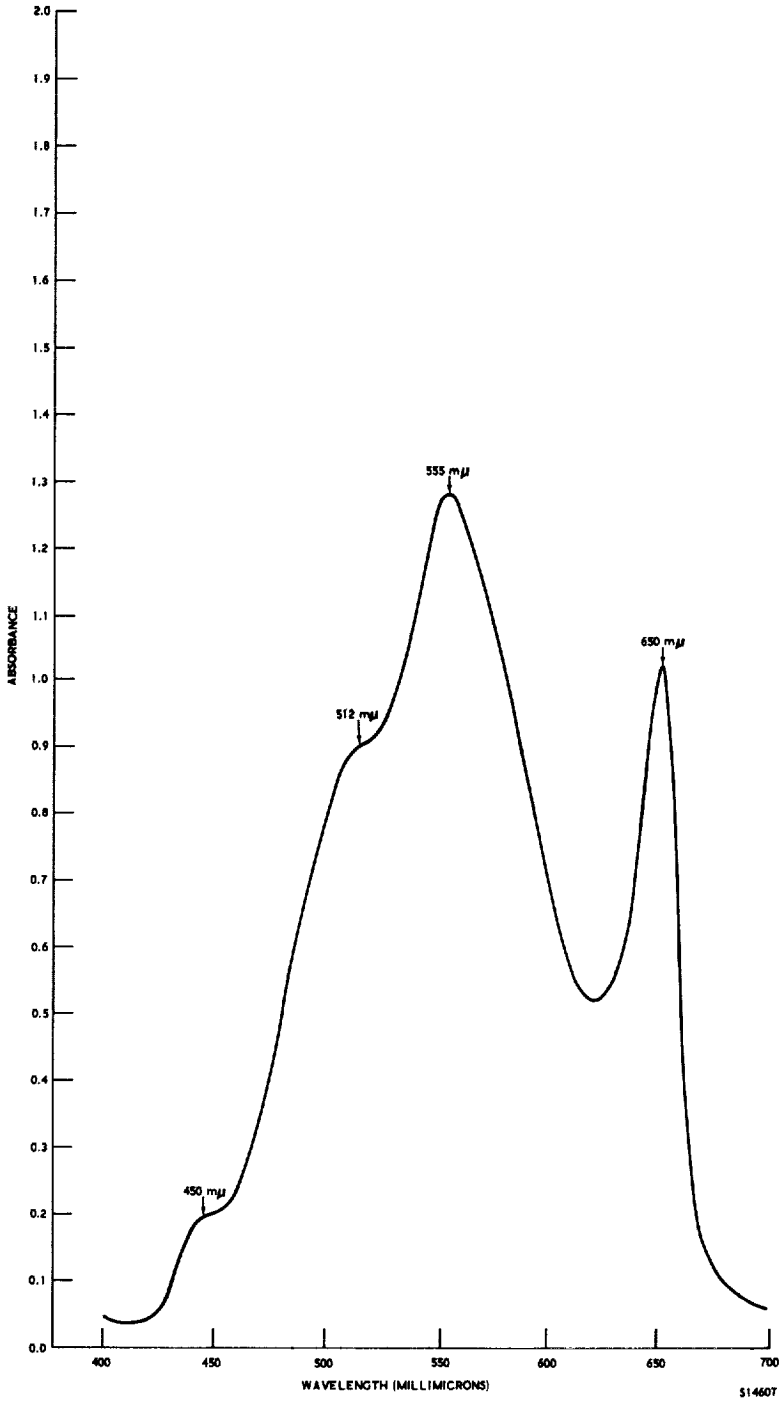


FIGURE 6. ABSORPTION SPECTRUM OF AQUEOUS DYE SOLUTION CONTAINING MAGNESIUM CHLORIDE.

The dye was $4 \times 10^{-5}$M and the $MgCl_2$ was $4.2 \times 10^{-3}$M. Measurements were made in a 1.0 cm cell against a water blank.

maximum to shorter wave lengths. Therefore, it is reasonable to expect that the formation of higher dye aggregates will be greatly affected by the concentration of negative ions. The presence of negative ions should favor aggregation and thus a change in the position of the absorption maximum of the dye to 650 and/or 450 mμ.

The amounts of electrolyte necessary to induce aggregation were determined by adding solutions containing various quantities of electrolyte to a buffered solution of the dye. The absorption spectrum of the solution was then determined against a water blank, and when changes in the spectrum indicating aggregation were observed, the electrolyte concentration was noted. Under these circumstances, increases in aggregation were always indicated by the formation of a J-band in the 650 mμ region of the spectrum when an appropriate amount of electrolyte was present. There was also often an additional absorption maximum in the region 555-585 mμ. Figure 6 shows a typical absorption spectrum obtained in the presence of sufficient electrolyte to cause aggregation. Table V gives the approximate minimum electrolyte concentration which was effective in causing the dye to form a J-band. It will be seen that small amounts of the electrolytes are required, and further, that their potency is dependent upon the charge of the negative ion. The effects of the electrolytes seem to be quite similar to their precipitating action on colloids[17].

It was found that in the presence of 10 percent ethanol, the salt concentration required to induce aggregation, as evidenced by J-band formation, was increased about tenfold. This may be due to the greater solubility of the dye in alcohol which gives rise to increased dispersion and decreased interaction of the dye molecules.

### 3.3.2 PROTEINS AND PROTEIN DERIVATIVES

It has already been shown that the addition of some proteins to the dye solution gives rise to the formation of new maxima in the absorption spectrum of the dye. The manner in which a number of different proteins interact with the dye is therefore of interest. This, in turn, makes the reaction of the dye with the constituents of proteins, e.g., peptides and amino acids, important for understanding some of the factors which influence the reaction of the dye with proteins. In order to obtain data which could be useful in analyzing the reaction of the dye with proteins, the influence of a large number of proteins, peptides and amino acids on the absorption spectrum of the dye was ascertained.

Thirty-four proteins were investigated. The influence of the proteins on the absorption spectrum of the dye is summarized in Table VI.

TABLE V

MINIMUM ELECTROLYTE CONCENTRATION EFFECTIVE IN CAUSING AGGREGATION OF THE DYE

(Aggregation was detected by the formation of a J-band. The dye concentration was $4 \times 10^{-5}$M and the solutions were buffered at pH 7.0 in 0.017M cacodylic acid buffer.)

| Electrolyte | Concentration (mM) | Elecrolyte | Concentration (mM) |
|---|---|---|---|
| $AlNH_4SO_4$ | 0.06 | $MgCl_2$ | 1.70 |
| $(NH_4)_2CrO_4$ | 0.08 | $CaCl_2$ | 1.70 |
| $K_2SO_4$ | 0.17 | KCl | 4.0 |
| $Na_2SO_4$ | 0.17 | NaCl | 4.0 |
| $MgSO_4$ | 0.17 | $Na_2CO_3$ | 4.0 |
| $ZnSO_4$ | 0.17 | $CuBr_2$ | > 8.0 |
| $CuSO_4$ | 0.17 | $Fe(NO_3)_3$ | >17.0 |
| $Fe_2(SO_4)_3$ | 0.17 | $Cu(NO_3)_2$ | >17.0 |
| $(NH_4)_2SO_4$ | 0.17 | $Ca(NO_3)_2$ | >17.0 |
| $KH_2PO_4$-$K_2HPO_4$ | 0.40 | $NaNO_3$ | > 17.0 |
| $Na_2HPO_4$ | 0.40 | $KNO_3$ | > 17.0 |
| $FeCl_3$ | 0.40 | | |
| $ZnCl_2$ | 1.70 | | |

TABLE VI

NEW ABSORPTION MAXIMA FORMED BY DYE
IN THE PRESENCE OF TRACE AMOUNTS OF PROTEIN

(The absorption spectrum of a $2 \times 10^{-5}$M solution of the dye in the presence of 0.002 percent protein was determined using a $2 \times 10^{-5}$M solution of the dye as a blank. All solutions were buffered in 0.001M tris buffer, pH 8.8, and measurements were made in 1.0 cm cells.)

| Protein | Commercial Sources** | Wave Lengths of New Maxima(mμ) |
|---|---|---|
| Ribonuclease, Oxidized, Bovine Pancreas | Mann | 650 |
| Oxytocin, Pituitary (Heterogeneous Sources) | Calbio | 620 |
| Fibrinogen, Bovine* | NBCo. | 643, 600 |
| Urease, Jack Bean Meal* | Calbio | 626, 570 |
| Alpha Globulin, Bovine Fraction IV | NBCo. | 650, 570 |
| Beta Globulin, Bovine Fraction III | NBCo. | 650, 570 |
| Acetylcholine Esterase, Bovine Erythrocytes | Mann | 637, 570 |
| Carbonic Anhydrase | Calbio | 642, 580 |
| Casein | Calbio | 646, 560 |
| Plasminogen, Bovine Fraction III | NBCo. | 642, 570 |
| Gelatin | Knox | 650, 580 |
| Pepsin, Swine gastric mucosa | Worth | 615, 559 |
| Pepsinogen, Swine gastric mucosa | Worth | 620, 558, 480 |
| Alpha Lipoprotein, Bovine | Calbio | 637, 570, 480 |
| Beta Lipoprotein, Bovine Fraction III-O | Calbio | 630, 560, 480 |
| Beta Lactoglobulin, Bovine | NBCo. | 646, 560, 480 |
| Myoglobin, Horse Heart | NBCo. | 640, 600, 570 |
| Glycoprotein, Bovine Fraction VI | NBCo. | 650, 600, 570 |
| Deoxyribonuclease, Bovine Pancreas | Calbio | 650, 600, 570, 450 |
| Cellulase, Fungal* | Calbio | 600, 570 |
| Pectinase, Fungal* | Calbio | 600, 570 |
| Follicle Stimulating Hormone, Porcine | Calbio | 600, 570 |
| Beta Glucuronidase, Bovine Liver | Mann | 600, 556 |
| Glutenin* | NBCo. | 600, 570 |
| Insulin, Bovine Pancreas* | Calbio | 600, 555, 480 |
| Trypsin, Bovine Pancreas* | Worth | 570 |

(continued)

TABLE VI (continued)

| Protein | Commercial Sources** | Wave Lengths of New Maxima(mμ) |
|---|---|---|
| Albumin, Bovine | NBCo. | 480 |
| Albumin, Human Fraction V | Calbio | 480 |
| Albumin, Egg* | NBCo. | 480 |
| Alpha Chymotrypsin, Bovine Pancreas | Worth | 480 |
| Hemoglobin, Human | NBCo. | 480 |
| Somatotrophic Hormone, Porcine | Calbio | 480 |
| Ribonuclease, Native, Bovine Pancreas | Mann | None |
| Carboxypeptidase, Pancreas | Calbio | None |

* These solutions were saturated and contained considerably less than 0.002 percent protein.

** Abbreviations used in the table are as follows:
NBCo. -- Nutritional Biochemicals Corp.
Calbio -- California Corp. for Biochemical Research
Mann -- Mann Research Laboratories, Inc.
Worth -- Worthington Biochemical Corp.

The results were quite dependent upon the particular protein used, and only in the case of carboxypeptidase and native ribonuclease did the addition of protein fail to cause the formation of new absorption maxima. The changes occurred with trace amounts of protein and the new maxima were always found at about 480, 570, 600 or 650 mμ. It appears that for a great many of the protein-dye complexes the exact wave lengths of the induced maxima may be characteristic of the protein.

The effects of thirty-seven different peptides on the absorption spectrum of the dye were determined. Only glycyl DL methionine produced changes in the dye spectrum at a concentration of less than 0.02 percent. However, at higher concentrations (0.1 percent) most of the peptides caused alterations in the absorption spectrum. The wave lengths of new maxima which were observed with a peptide concentration of 0.1 percent are recorded in Table VII. The alterations, as in the case of the proteins, involved combinations of increases in absorbance at wave lengths of about 480, 570, 600 and 650 mμ. However, in the vast majority of the cases, the absorbances of the induced maxima were not very great and the main effect was the formation of a new maximum at about 560 to 570 mμ, which indicates that the effect was one of deaggregation rather than aggregation of the dye. The occurrence of hydrophobic or hydrophilic side-chains and the ionizability of the hydrophilic side-chains in the peptides did not appear to have any consistent influence upon the nature of the changes in the absorption spectrum. However, the sequence of the amino acids in the peptide was of considerable importance; e.g., glycyl leucine caused the formation of an intense absorption peak at 650 mμ, whereas leucyl glycine was without effect. Likewise, glycyl alanine induced a new absorption peak at 540 mμ, but alanyl glycine did not influence the absorption spectrum.

The effect of 0.1 percent solutions of forty different amino acids or amino acid derivatives on the absorption spectrum of the dye was investigated in unbuffered solutions, 0.017M cacodylic acid buffered solutions, pH 7.0 and 0.001M tris buffered solutions, pH 8.8. New maxima(645 and 540 mμ) were observed only in the case of L-meso lanthionine. This amino acid has a structure which is not typical of most amino acids. It can be considered to be a diamino acid composed of two molecules of alanine linked by a sulfur bridge. As such, it has some of the attributes of a dipeptide and might be expected to behave more like a peptide than a simple amino acid. Its ability to induce new maxima in the dye spectrum is in agreement with this idea.

A simple amino acid has about twice as many ionizable amino and carboxylic groups as an equal weight of its dipeptide. Thus, if the number of ionizable, anionic sites available determines the effectiveness of a substance to change the aggregation state of the dye, it would appear that, on a weight basis, simple amino acids should be more effective agents than peptides. However, this is not the case and in fact many of the peptides

TABLE VII

NEW ABSORPTION MAXIMA FORMED BY DYE IN THE PRESENCE OF PEPTIDES

(The absorption spectrum of a $2 \times 10^{-5}$ or $4 \times 10^{-5}$M solution of the dye in the presence of 0.1 percent of each peptide was determined in a 1.0 cm cell using the aqueous dye solution or distilled water as a blank. Measurements were made in unbuffered solutions and in solutions buffered with 0.001M, pH 8.8 tris buffer, or 0.017M, pH 7.0 cacodylic acid buffer. All of the peptides were obtained from Nutritional Biochemical Corp.)

| Peptide | Wave Lengths of New Maxima (mμ) |
|---|---|
| D Leucyl Glycyl Glycine | 650, 600, 565 |
| DL Alanyl Glycyl Glycine+ | 650, 562 |
| DL Alanyl DL Phenylalanine* | 648, 585 |
| DL Alanyl DL Alanine | 650, 560, 482 |
| DL Alanyl DL Asparagine++ | 650, 537 |
| Glycyl L Asparagine+ | 650, 560 |
| Glycyl D Asparagine+ | 650, 554 |
| Glycyl Glycyl Glycine | 620, 560 |
| Glycyl DL Methionine* | 620, 565, 445 |
| Glycyl L Leucine | 612, 570 |
| Glycyl Glycine | 610, 565 |
| DL Alanyl DL Leucine | 610, 560 |
| DL Alanyl DL Norleucine | 610, 560 |
| DL Leucyl DL Phenylalanine | 610, 560 |
| Histidyl Histidine | 608, 565 |
| Glycyl DL Leucine | 605, 560 |
| Glycyl DL Phenylalanine | 605, 565 |
| Glycyl DL Norleucine | 605, 565 |
| Glycyl DL Valine | 605, 568 |
| Glycyl Glycyl Glycyl Glycine | 605, 570 |
| Glycyl L Tyrosine | 605, 560 |
| DL Leucyl Glycyl Glycine | 605, 560 |
| DL Leucyl DL Norvaline | 605, 560 |
| DL Alanyl DL Valine | 605, 560 |

(continued)

TABLE VII (continued)

| Peptide | Wave Lengths of New Maxima (mμ) |
|---|---|
| Glycyl L Tryptophane | 604, 560 |
| Glycyl DL Norvaline | 602, 565 |
| Glycyl Glycine · HCl | 600, 560 |
| D Leucyl Glycine | 600, 565 |
| DL Alanyl DL Norvaline | 600, 565 |
| Glycyl DL Alanine++ | 570, 540 |
| Glycyl DL Serine | None |
| DL Alanyl Glycine | None |
| DL Leucyl Glycine | None |
| D Leucyl L Tyrosine | None |
| L Leucyl L Tyrosine | None |
| DL Benzoyl Alanine | None |
| Benzoyl Glycine | None |

+ The indicated response was obtained only in the unbuffered and cacodylic acid buffered solutions.

++ The indicated response was obtained only in the cacodylic acid buffered solution.

* The peptide solution was saturated and probably contained considerably less than 0.1 percent peptide.

are effective agents in 0.02 percent solution, whereas none of the simple amino acids are effective even at concentrations of 0.1 percent. Both peptides and amino acids contain ionizable amino and carboxylic groups; however, the spatial relationship of the ionizable amino group to the ionizable carboxylic group in a simple amino acid is quite different from that in the peptide. In the peptide these groups are separated by a relatively large distance, while in the free amino acid they are adjacent. Perhaps the proximity of the cationic site to the anionic site is a factor which influences the effect of amphoteric substances on the aggregation or deaggregation of the dye.

With the exception of glycyl DL serine, the peptides which failed to cause changes in the absorption spectrum of the dye show one of two characteristics. They either contain a large aromatic structure or have glycine as the donor of the carboxylic end group. It is possible that the aromatic group hinders the close approach of the dye molecule to the peptide and thus the anionic site on the peptide is unable to influence the aggregation state of the dye. In regard to the apparently unique effect of the glycine entity in dipeptides, it is noteworthy that there is no side-chain on the alpha carbon of the peptide only when glycine acts as the donor of the carboxylic end group. Perhaps the absence of the side-chain permits the carboxylic and amino groups to approach each other more closely, and the influence of the anionic site on the dye molecule is counteracted by the adjacent cationic site. The fact that diketopiperazines can be readily formed indicates that the peptide chain is flexible enough to allow the amino and carboxylic groups to approach each other.

Since the proteins are far more effective, on a weight basis, than the peptides, the reaction of the dye with proteins appears to differ from that with the peptides. Compared with peptides, the terminal carboxylic groups of proteins are separated farther from the end amino groups. However, since there are so few terminal groups per mass of protein, it seems unlikely that this increased separation can be responsible for the greatly increased effectiveness of proteins to provide focal points for dye aggregation. Rather, it is generally recognized[1] that the effective sites are the anionic side-chains which are available along the backbone of the protein. Thus, it is possible that the effect of a particular protein on the dye spectrum is related to the type, spacing, and number of these anionic side-chains.

### 3.3.3 SYNTHETIC POLYPEPTIDES

The study of the reaction of synthetic polypeptides with the dye presents an opportunity to examine, in a more exact manner, the influence of various types of repeating side-chains on the changes in the absorption

spectrum of the dye. By choosing appropriate polypeptides where all the side-chains in any one polypeptide are the same, it is possible to ascertain the relationship of a particular repeating functional group to the changes in the absorption spectrum of the dye. The following long chain polypeptides dissolved in 0.017M cacodylic acid buffer, pH 7.0, were examined: (a) poly-L-lysine hydrobromide (MW 100,000-200,000), having ionizable cationic $H_2N(CH_2)_4$-side groups, (b) poly-L-aspartic acid (MW 5,000-10,000) with ionizable anionic $HOCO \cdot CH_2$-side groups, (c) poly-L-hydroxyproline (MW 50,000-100,000) with hydrophilic but non-ionizable $HOCH(CH_2)_2$-side chains, and (d) poly-L-proline (MW 25,000-50,000) having hydrophobic $H_2C(CH_2)_2$-side groups.

The presence of poly-L-hydroxyproline, poly-L-proline or poly-L-lysine hydrobromide at a concentration of 0.02 percent did not alter the absorption spectrum of the aqueous dye solution, whereas a 0.0002 percent solution of poly-L-aspartic acid produced a very intense absorption peak at about 530 mμ (Figure 7).

The great sensitivity of the dye to the presence of poly-L-aspartic acid indicates that the presence of a compound with repeating, regularly spaced, ionizable, anionic side-chains is especially conducive to the formation of an absorption peak at about 530 mμ. On the other hand, since poly-L-lysine is ineffective, it appears that regularly spaced, ionizable cationic side-chains do not influence the aggregation of the dye. In addition, since both poly-L-hydroxyproline and poly-L-proline are ineffective, hydrophobic or hydrophilic non-ionizable side groups apparently do not directly influence the aggregation of the dye and it appears that the cationic and anionic end groups present in these compounds are also not important in this respect.

Thus, it appears that the anionic carboxylic side-chains exert a great influence on the aggregation of the dye, but it is also apparent that the structural arrangement is of great importance. This is indicated by the fact that aspartic acid, even at a concentration of 0.1 percent, does not influence the absorption spectrum of the dye, whereas poly-L-aspartic acid is very effective at concentrations of only 0.0002 percent.

### 3.3.4 DEOXYRIBONUCLEIC ACID (DNA), RIBONUCLEIC ACID (RNA) AND DERIVATIVES

Since the regularly spaced anionic side-chains of poly-L-aspartic acid are extremely effective in altering the aggregation of the dye, other substances having regularly spaced ionizable anionic groups may also be potent agents. The nucleic acids possess these attributes and they are also key building blocks in biological systems. Therefore, the interactions

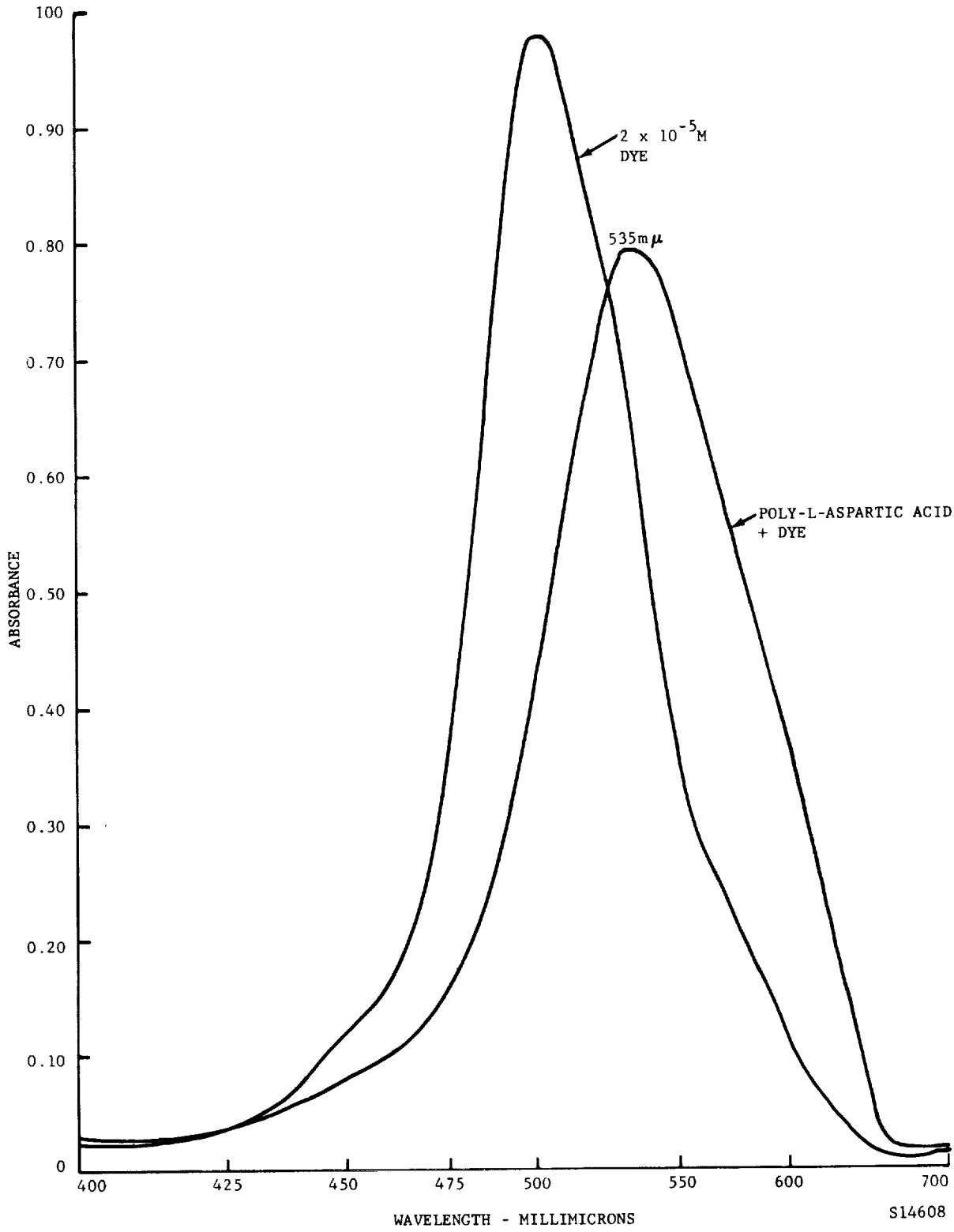


FIGURE 7. EFFECT OF POLY-L-ASPARTIC ACID ON THE ABSORPTION SPECTRUM OF THE DYE.

The dye was 2 x $10^{-5}$M and the poly-L-aspartic acid 0.0002 percent. The mixture was buffered at pH 7.0 in 0.017M cacodylic acid buffer, and measurements were made in a 1.0 cm cell against a water blank. The aqueous dye spectrum is shown for comparison.

of the dye with salmon sperm DNA (Calif. Corp. for Biochem. Research), calf thymus DNA (Worthington Biochem. Corp.) and yeast RNA (Calif. Corp. for Biochem. Research) were investigated.

DNA samples were prepared by dissolving, with gentle agitation at 4°C, weighed amounts of DNA in 0.05M cacodylic acid buffer, pH 7.0, containing 1.0 mM $MgCl_2$. The DNA solution was then centrifuged at 19,000 times gravity and 4°C for 60 minutes and the supernatant solution decanted from the sediment. Aliquots of the supernate were diluted with the buffer solution to give a series of solutions having different DNA concentrations. A weighed amount of yeast RNA was dissolved in buffer in the same manner as the DNA, but the preparation was not centrifuged.

Each solution of DNA and RNA was mixed with an equal amount of $8 \times 10^{-5}$M aqueous dye solution and the absorption spectra were determined using water as a reference. Both DNA samples induced new intense absorption peaks at 570 mμ (Figure 8). The calf thymus DNA-dye mixture formed a suspension of blue fiber-like particles which precipitated after standing for several hours. On the other hand, the salmon sperm DNA-dye mixture formed a clear blue solution and no precipitate was evident. In contrast to the 570 mμ peak induced by DNA solutions, RNA caused the formation of a new intense absorption peak at 535 mμ (Figure 8). The exact concentrations of the DNA and RNA solutions were not known, since the degree of hydration and the losses due to centrifugation were not ascertained. However, based on the weight of material used, a 0.0002 percent solution of DNA or RNA produced new absorption peaks at 570 mμ or 535 mμ.

Because of the marked interaction of DNA and RNA with the dye, it was of interest to investigate the interaction of the dye with their constituent parts, the pyrimidine and purine bases, nucleosides and nucleotides. Each substance was tested initially at a concentration of 0.02 percent in 0.017M, pH 7.0 cacodylic acid buffer. Only the nucleotides altered the absorption spectrum of the dye. In every case an intense absorption peak was formed at about 650 mμ and additional peaks were present, in the 570 or 540 mμ regions of the spectrum. Table VIII lists the nucleotides tested, and indicates the wave lengths of new maxima and the minimum nucleotide concentrations which caused alterations in the absorption spectrum of the dye. The minimum effective concentration for the nucleotides varied considerably. However, in each case, the 3'(2') compounds were considerably more effective than their corresponding 5' nucleotides, and within groups having the phosphoric acid at the same position, the purine containing nucleotides were more effective than the pyrimidine containing nucleotides. Thus it appears that the influence of the nucleotide on the dye spectrum is more dependent upon spatial considerations than the nature of the functional groups which are present. This is evidenced by the fact that nucleotides having similar

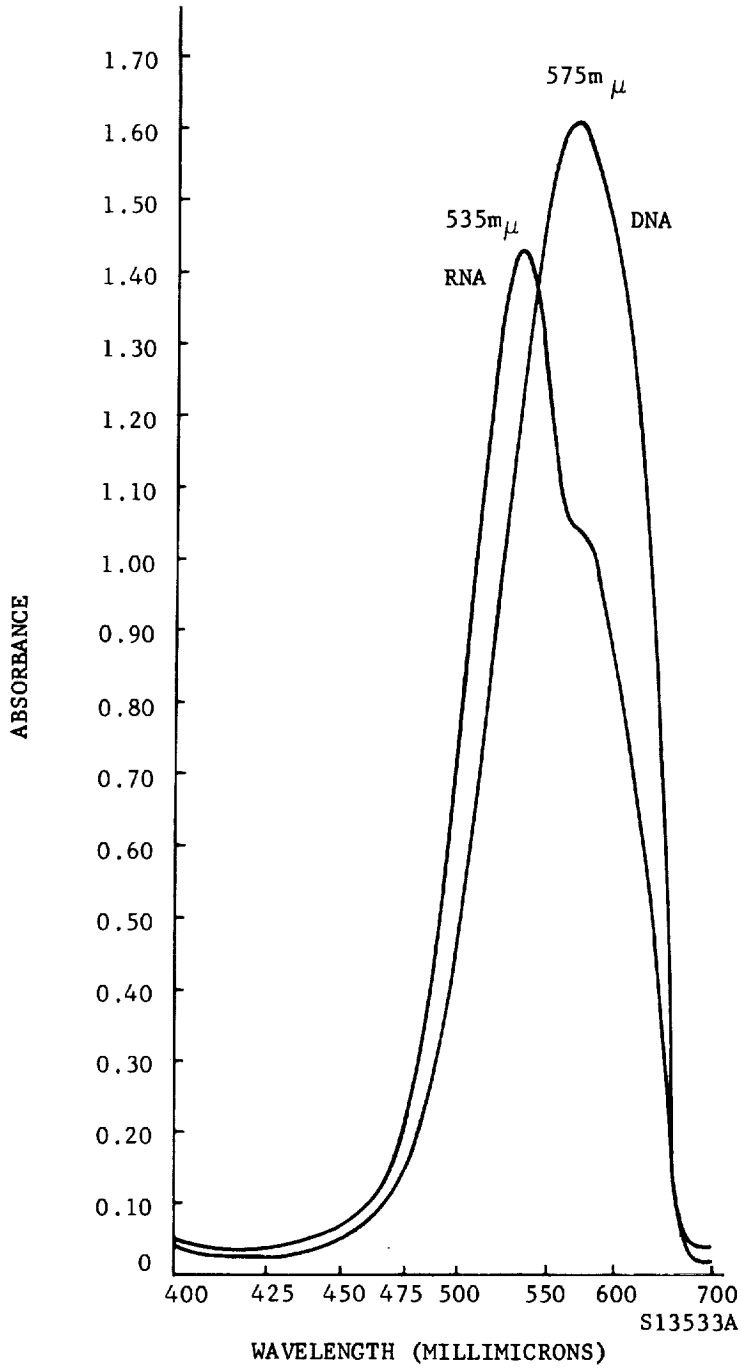


FIGURE 8. ABSORPTION SPECTRA OF DNA- AND RNA-DYE COMPLEXES

Solutions of DNA and RNA were prepared in 0.017M Cacodylic Acid Buffer, pii 7.0 containing 0.5 mM $MgCl_2$. The DYE is 4 x $10^{-5}$M and the DNA and RNA concentrations are about 0.007 percent. Measurements were made against water in a 1.0 CM cell.

TABLE VIII

NEW ABSORPTION MAXIMA FORMED BY DYE IN THE PRESENCE OF NUCLEOTIDES

(The absorption spectrum of a $4 \times 10^{-5}$M solution of the dye in the presence of various amounts of nucleotides was determined using water as a blank. All solutions were buffered at pH 7.0 in 0.017M cacodylic acid buffer and measurements were made in 1.0 cm cells.)

| Nucleotide | Wave Lengths of New Absorption Maxima (mμ) | Minimum Effective Nucleotide Concentration (moles/liter) |
|---|---|---|
| 3'(2') Adenylic Acid | 660, 585, 535 | $1.3 \times 10^{-5}$ |
| 3'(2') Guanylic Acid | 648, 540 | $1.2 \times 10^{-5}$ |
| 3'(2') Cytidylic Acid | 655, 530 | $6 \times 10^{-5}$ |
| 3'(2') Uridylic Acid | 655, 600, 530 | $6 \times 10^{-5}$ |
| 5' Deoxyadenylic Acid | 655, 535 | $5.6 \times 10^{-5}$ |
| 5' Deoxyguanylic Acid | 660, 575, 530 | $6 \times 10^{-5}$ |
| 5' Deoxycytidylic Acid | 655, 580, 530 | $1.5 \times 10^{-4}$ |
| 5' Thymidylic Acid | 650, 580, 530 | $1.2 \times 10^{-4}$ |
| Cytidine · $\frac{1}{2}H_2SO_4$ | 652, 575 | $3.4 \times 10^{-4}$ |

structures, but different functional groups (e.g., adenylic acid and guanylic acid) affect the dye at about the same concentrations, whereas nucleotides having different structures, but the same functional groups (e.g., adenylic acid and cytidylic acid) do not. Since the only difference between the nucleosides, which do not induce new absorption bands, and the nucleotides is the presence of a phosphate group in the latter, it appears that this group is responsible for the formation of the new absorption maxima. At pH 7, the phosphate group is negatively charge; therefore, as in the case of the inorganic salts, anionic sites appear to be potent factors in the formation of new absorption maxima. However, the similarity with the inorganic anions is not complete, since on a molar basis the 3'(2') nucleotides are 10 to 40 times more effective than $KH_2PO_4$-$K_2HPO_4$. Thus, as in the case of the proteins and their constituents, the nucleic acids and their constituents increase in effectiveness as their complexity and structural organization increase. That is to say, the nucleic acids are more effective than the nucleotides which are in turn more effective than inorganic phosphate.

### 3.3.5 CARBOHYDRATES

The results of the studies on proteins and nucleic acids strongly indicate that anionic sites and a highly organized structure are important factors which influence the ability of a substance to cause a change in the aggregation of the dye. The carbohydrates include a larger number of substances which vary greatly in regard to complexity and the occurrence of anionic sites. Appel and Scheibe[6] have observed marked changes in the absorption spectrum of N,N'-diethyl-pseudocyanine iodide in the presence of small amounts of heparin, chondroitin sulfate and hyaluronic acid. All of these substances are large complex polysaccharides which possess numerous anionic sites. In view of this demonstrated effect of polysaccharides on the absorption spectrum of a cyanine dye, it was of interest to examine the interaction of a number of carbohydrates with the dye used in the present experiments. Forty-two carbohydrates were tested in unbuffered solutions and in solutions buffered at pH 7.0 with 0.017M cacodylic acid. None of the mono-, di- or trisaccharides altered the absorption spectrum of the dye in any manner, but some of the polysaccharides caused marked changes. Long-chain polysaccharides, such as inulin and dextrin, which do not possess anionic sites had no effect. On the other hand, heparin, polygalacturonic acid, agar, chondroitin sulfate, alginic acid, hyaluronic acid and carboxy-methyl cellulose ether, all of which have large numbers of anionic groups caused the formation of new absorption maxima. In most cases, these new maxima appeared in the presence of 0.0002 to 0.00006 percent of the substance tested and each macromolecule affected the dye spectrum in a somewhat different way. As an example of the changes observed, Figure 9 shows the effect of a trace amount of alginic acid on the absorption spectrum of the dye.

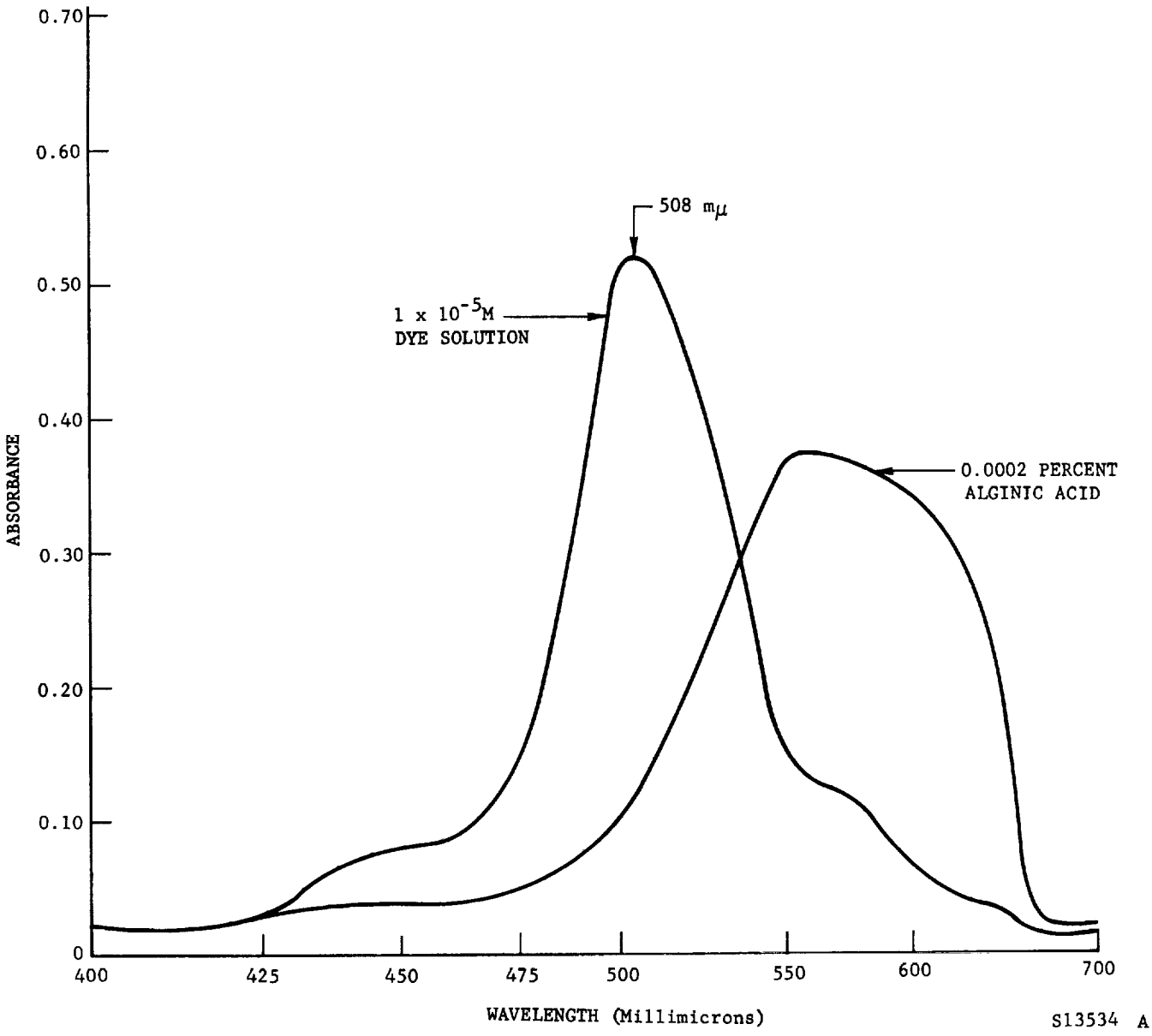


FIGURE 9. ABSORPTION SPECTRUM OF ALGINIC ACID-DYE COMPLEX

The solution was buffered in 0.017M, cacodylic acid buffer pH 7.0, the dye concentration was 1 x $10^{-5}$M and the alginic acid concentration 0.0002 percent. Measurements were made against water in a 1.0 CM cell. The absorption spectrum of a 1 x $10^{-5}$M dye solution in buffer is shown for comparison.

These results again emphasize the importance of structure and the occurrence of anionic sites as factors in the interaction of the dye with macromolecules. However, detailed information on the relationships of the nature of the anionic sites and the configuration of the polysaccharide to the changes in the dye spectrum must await further investigation.

## 3.4 INFLUENCE OF ENVIRONMENT ON THE NATURE OF THE SPECTRAL CHANGES INDUCED BY TEST SUBSTANCES

### 3.4.1 ALTERATIONS IN pH

It has been shown that the formation of new absorption maxima in the presence of macromolecules is a function of the sign of the electric charge on the macromolecule. Thus it is reasonable to expect that a change in the sign of the electric charge will cause extensive changes in the influence of the macromolecule on the dye. Proteins possess both anionic and cationic sites and therefore are amphoteric substances. Thus it is possible, by altering the pH, to change their electrical nature from anionic to cationic and this should result in readily detectable changes in the interaction of the dye with the protein.

To examine the influence of pH on the dye-protein complex, small amounts of 1N HCl or 1N NaOH were added to aqueous mixtures of dye plus protein. The pH and absorbance, at the wave length characteristic of the dye-protein complex, were determined after each addition of HCl or NaOH and the data obtained is shown in Figure 10. For every protein tested, a decrease in pH resulted in a decrease in the intensity of the induced dye absorption peak. However, a steady decrease in absorbance was not observed. Instead, the absorbance remained relatively constant over a considerable pH range and then a rapid decrease was evident at lower pHs. The pH values over which this rapid decrease in absorbance occurred were characteristic of the protein being tested and varied from 8.6 to 7.6 for cytochrome-c to 3.1 to 1.7 for urease.

These results can be explained as being due to changes in the net charge on the protein. A decrease in pH causes the net negative charge on a protein to decrease. At a pH characteristic of the protein, the net charge becomes zero and as the pH is lowered still more the net charge becomes positive. Thus with a decrease in pH it is reasonable to expect that the cationic-dye will be less tightly bound to the protein. This will result in detachment of the dye from the protein, which will cause deaggregation of the dye and a decrease in absorbance at the wave length characteristic of the dye-protein complex. When the pH becomes

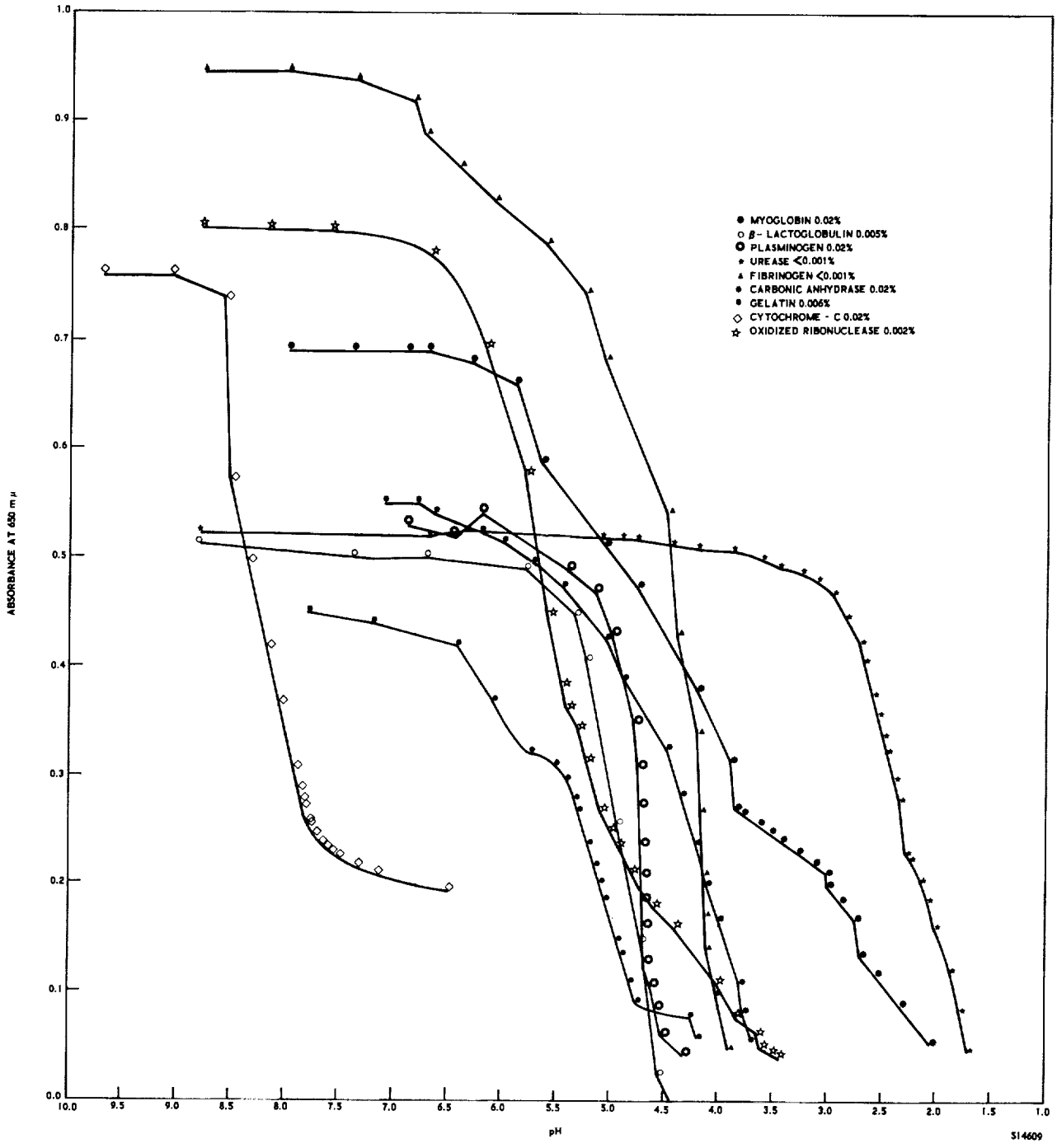


FIGURE 10. THE EFFECT OF pH ON THE ABSORBANCE OF DYE-PROTEIN COMPLEXES

Aqueous solutions of the proteins were mixed with equal volumes of $8 \times 10^{-5}$M dye solution. Separate aliquots were then titrated with either 1N NaOH or 1N HCl and the absorbance at 650 mμ was determined at the pHs indicated using a $4 \times 10^{-5}$M dye solution as the blank.

sufficiently acid all of the dye will be removed from the protein and new dye absorption maxima will no longer be observed. It is noteworthy that in the proteins tested the pH corresponding to this latter situation is characteristic of the protein and is always less than the published isoionic point. This indicates that even when the net charge on the protein is positive, the dye is still able to interact with the ionizable anionic sites.

### 3.4.2 LENGTH OF REACTION TIME

At a given time after mixing the reactants, the intensity of the characteristic absorption maximum of a dye-macromolecule complex or a dye aggregate induced by a given substance will be a function of the overall reaction rate of the processes taking place. Thus, if the rate constants are different for specific substances, the time to reach equilibrium conditions will vary with the substance tested.

The length of time required to reach equilibrium conditions at 24°C was determined for solutions of the dye with $\beta$-lactoglobulin, gelatin, myoglobin, carbonic anhydrase, salmon sperm DNA (stabilized with 1 mM $MgCl_2$), thymidylic acid and $KH_2PO_4$. Each test substance was mixed with an equal volume of $8 \times 10^{-4}$M dye solution and the absorbance at the characteristic absorption maxima for each of these preparations was determined as a function of time after mixing.

The data presented in Figure 11 show that a considerable length of time is required for dye-protein mixtures to reach equilibrium conditions. On the other hand, solutions of the dye with DNA, thymidylic acid or $KH_2PO_4$ reached equilibrium conditions in a very short time. Thus it appears that the rate constants for the reactions with protein are different from those for the reactions which take place in the presence of DNA, thymidylic acid or $KH_2PO_4$. Furthermore, the results indicate that if one wishes to determine the maximum absorbance of a dye-protein complex, it is necessary to wait long enough to be sure that equilibrium has been achieved.

### 3.4.3 CHANGES IN TEMPERATURE

Since the adsorption of dye molecules on macromolecules and the interaction of the dye molecules with each other to form aggregates are equilibrium conditions, it can reasonably be expected that temperature will influence the equilibrium and cause changes in the absorbance of dye-macromolecule complexes or aggregated dye molecules. Indeed, the data previously presented on the effect of temperature on aqueous dye solutions show that

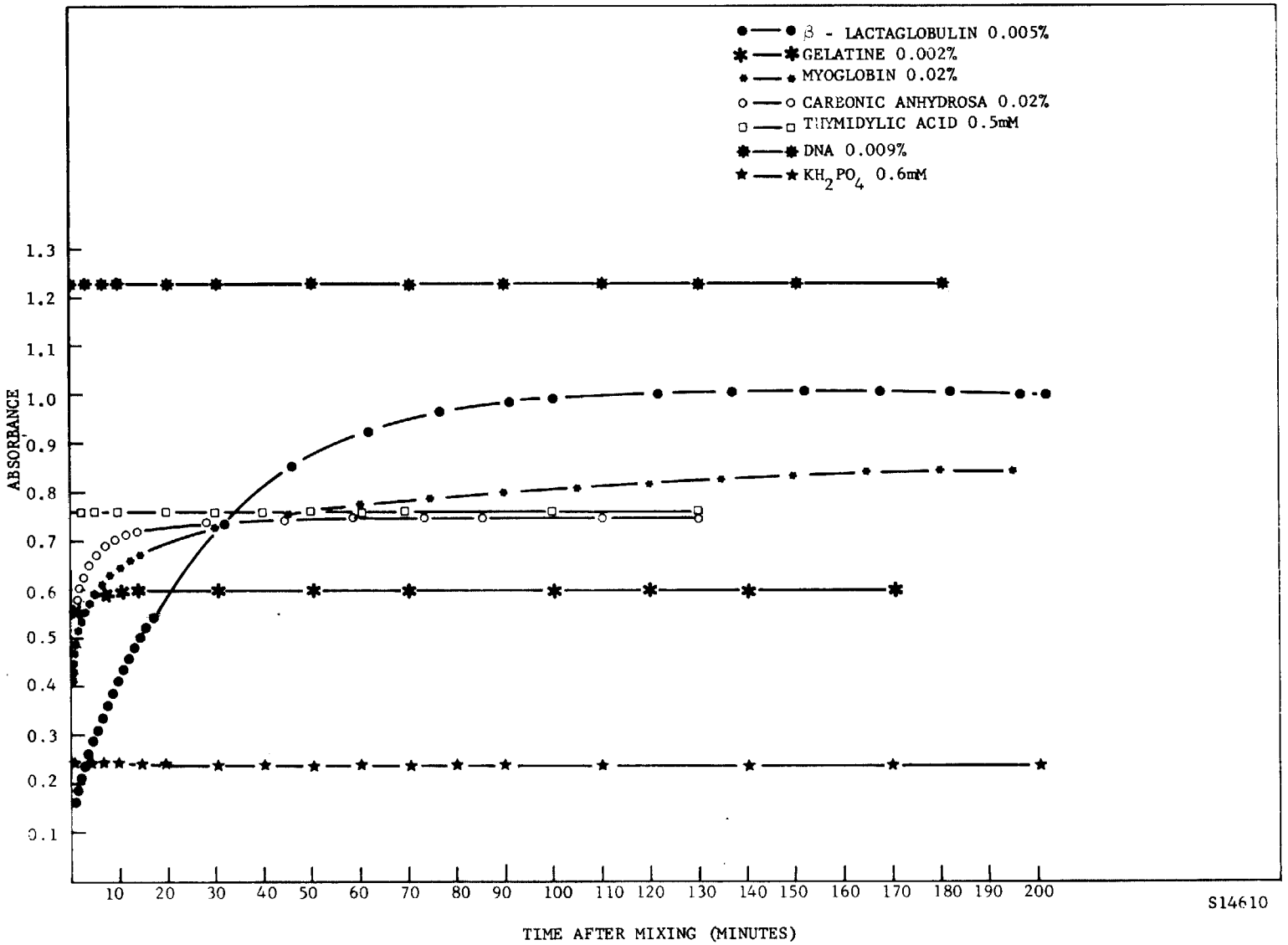


FIGURE 11. RATE OF FORMATION OF DYE AGGREGATES IN THE PRESENCE OF VARIOUS SUBSTANCES

The dye concentration was $4 \times 10^{-5}$M and except for the case of DNA, the absorbance was determined against a dye blank at 650 mμ . For DNA the absorbance was determined at 575 mμ with water as the blank.

elevated temperatures cause deaggregation of the dye, and a decrease in the absorbance at the wave length characteristic of the aggregated species. Thus temperature appears to be an important factor in the aggregation of the dye and the effect of temperature on the absorbance at the characteristic wave length of the dye-macromolecule complexes and dye aggregates is of interest.

To investigate the influence of temperature, mixtures of dye with β-lactoglobulin, gelatin, DNA (partially denatured), thymidylic acid or $KH_2PO_4$ were used as test materials. Separate aliquots of the test substances and the dye were adjusted to the temperatures indicated in Figure 12, mixed together, kept at the indicated temperature until equilibrium was reached, and the absorbance determined. The results are shown in Figure 12.

As evidenced by the absorbance, the aggregation of the dye in the presence of $KH_2PO_4$ or thymidylic acid is favored by low temperatures. At 7°C a very intense absorption peak was formed in the presence of $KH_2PO_4$, but with increasing temperature the absorbance diminished and, at 39°C, $KH_2PO_4$ no longer induced the dye to aggregate. Rather surprisingly, this was also true when the concentration of $KH_2PO_4$ was increased 10 fold. Likewise, an intense absorption peak was formed at 7°C in the presence of thymidylic acid and with increasing temperature the absorbance decreased until at 47°C the peak at 660 mμ was no longer evident. The influence of temperature on the absorbance of dye-macromolecule complexes was quite different from that found for $KH_2PO_4$ and thymidylic acid. Temperature had very little influence on the absorbance of the dye-DNA complex. The absorbance of the dye-DNA complex increased at 535 mμ and decreased at 575 mμ with increasing temperature. This indicates that the changes in absorbance which did take place were due to denaturation of the DNA (see Section 3.5.1) rather than instability of the dye-DNA complex. In order to reduce the effects of denaturation, the highest temperature tested in the case of β-lactoglobulin was 36°C. However, within the limited temperature range examined the absorbance increased with increasing temperature. The effect of temperature on the absorbance of the dye-gelatin complex was rather inconsistent in that temperatures both above and below 30°C caused increases in the absorbance. However, these increases were not large and, in general, it appeared that the absorbance of the gelatin-dye complex was not greatly influenced by temperature.

The data obtained in these experiments, strongly indicate that the dye-macromolecule complexes have a much greater thermal stability than do the dye aggregates found in the presence of $KH_2PO_4$ or thymidylic acid.

### 3.4.4 EFFECT OF DYE-PROTEIN STOICHIOMETRY

The effect of macromolecule concentration on the absorbance of the dye-macromolecule complex was investigated for gelatin, β-lactoglobulin,

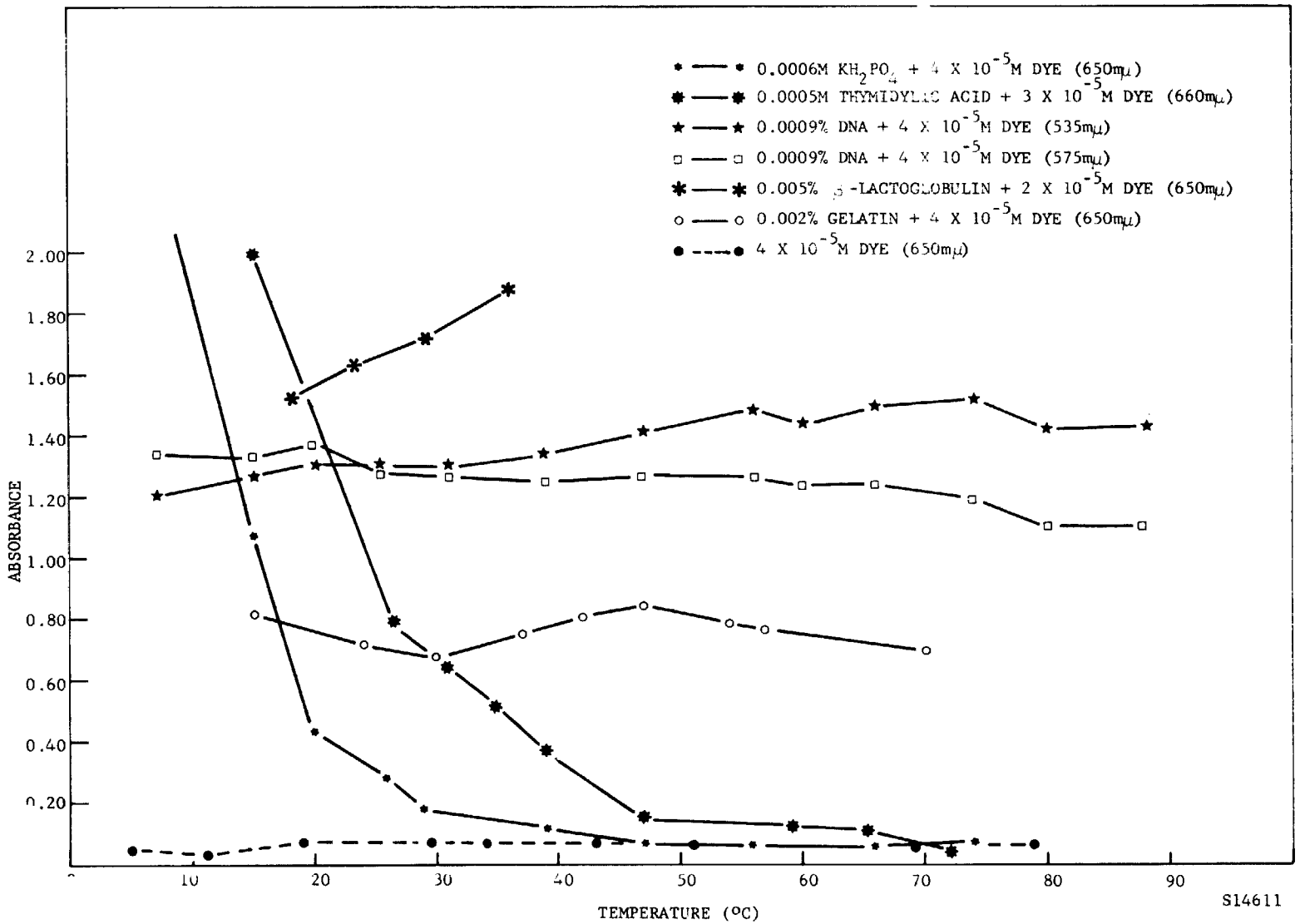


FIGURE 12. EFFECT OF TEMPERATURE ON THE ABSORBANCE OF DYE-MACROMOLECULE COMPLEXES AND DYE AGGREGATES INDUCED BY $KH_2PO_4$ AND THYMIDYLIC ACID

Measurements were made in 1.0 cm cells, at the wave lengths and dye concentrations indicated in the legend, using water as the blank

salmon sperm DNA, and oxidized ribonuclease (oxRNAse). Solutions containing 4 x $10^{-5}$M dye and the amounts of these substances indicated in Figure 13 were prepared. The protein-dye mixtures were buffered at pH 7.0 with 0.017M cacodylic acid buffer, but the DNA solutions were not buffered. The mixtures were allowed to come to equilibrium, the absorption spectra determined and the absorbance at the wave lengths characteristic of the dye-macromolecule complexes were obtained. The results are shown in Figure 13.

The data show that a macromolecule concentration of less than one microgram per ml causes the formation of a new absorption peak and the absorbance at this peak increases in an approximately linear manner with the macromolecule concentration.

The effect of altering both the dye and protein concentrations was investigated only in the case of oxidized ribonuclease (oxRNAse) and DNA. Solutions of oxRNAse and dye were prepared as before except that dye concentrations of 4 x $10^{-5}$, 2 x $10^{-5}$, or 1 x $10^{-5}$M were used. The solutions were allowed to equilibrate, the absorption spectrum was determined and the absorbance at 650 mμ obtained. The molar ratio of dye to oxRNAse was computed in each case and the absorbance plotted as a function of these ratios. Four solutions of DNA were prepared as before and mixed with various dye solutions so that the concentration of the dye varied from 6 x $10^{-5}$M to 0.33 x $10^{-5}$M.

The data in Figure 14 show that at each dye concentration the absorbance increased with increasing oxRNAse concentration, but only when the dye to oxidized ribonuclease molar ratio was greater than 5.7. On the other hand, when the molar ratio was less than 5.7, the absorbance remained constant or decreased with increasing protein concentration. (At concentrations of 2 x $10^{-5}$ or 4 x $10^{-5}$M, it remained constant, and when the dye was 1 x $10^{-5}$M it decreased.) In the case of the 1 x $10^{-5}$M dye, the decrease in absorbance at the maximum was accompanied by a change in the shape of the absorption spectrum so that the peak was not as sharp, the absorbance was greater at shorter wave lengths, and a weak new absorption maximum appeared at 448 mμ (Figure 15). This new absorption maximum probably represents a dye aggregate which is smaller than the one responsible for the 650 mμ peak. The dotted lines in Figure 14 connect points representing mixtures which contain equal protein concentrations and have a dye to protein ratio of 5.7 or more. From an inspection of the resulting curves it is readily apparent that when the protein concentration is held constant, the absorbance of the dye-oxRNAse complex increases with increasing dye concentration. This indicates that the equilibrium is not exceptionally far on the side of complex formation.

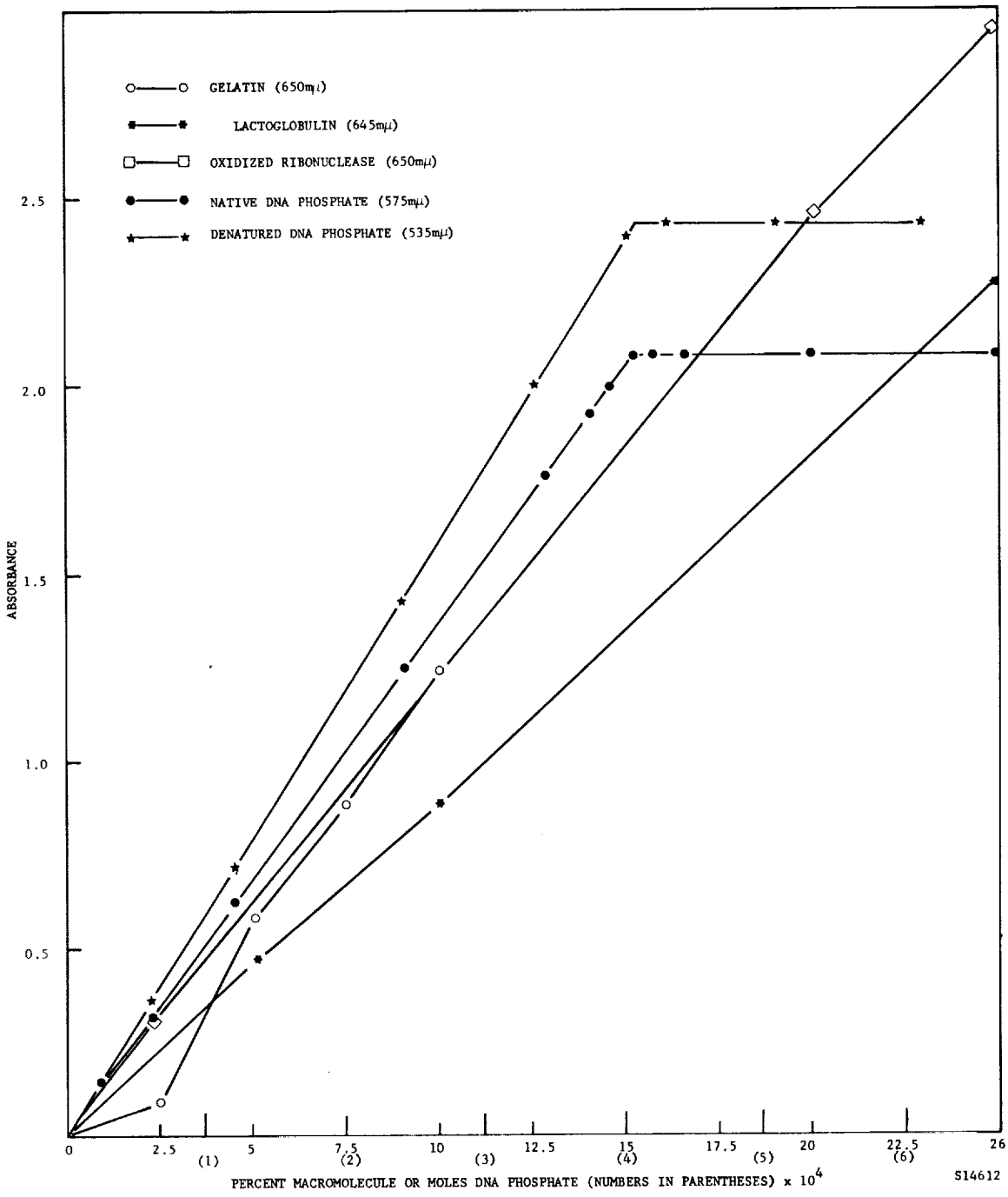


FIGURE 13. EFFECT OF PROTEIN CONCENTRATION ON THE ABSORBANCE OF DYE PROTEIN MIXTURES

The measurements were made in 1.0 or 0.1 cm cells at the wave length indicated in the legend. All values are adjusted to a 1.0 cm path length and corrected for absorbance due to the free dye. The dye concentration was $4 \times 10^{-5}$M and the protein solutions were buffered at pH 7.0 with 0.017M cacodylic acid buffer. The DNA solutions were not buffered.

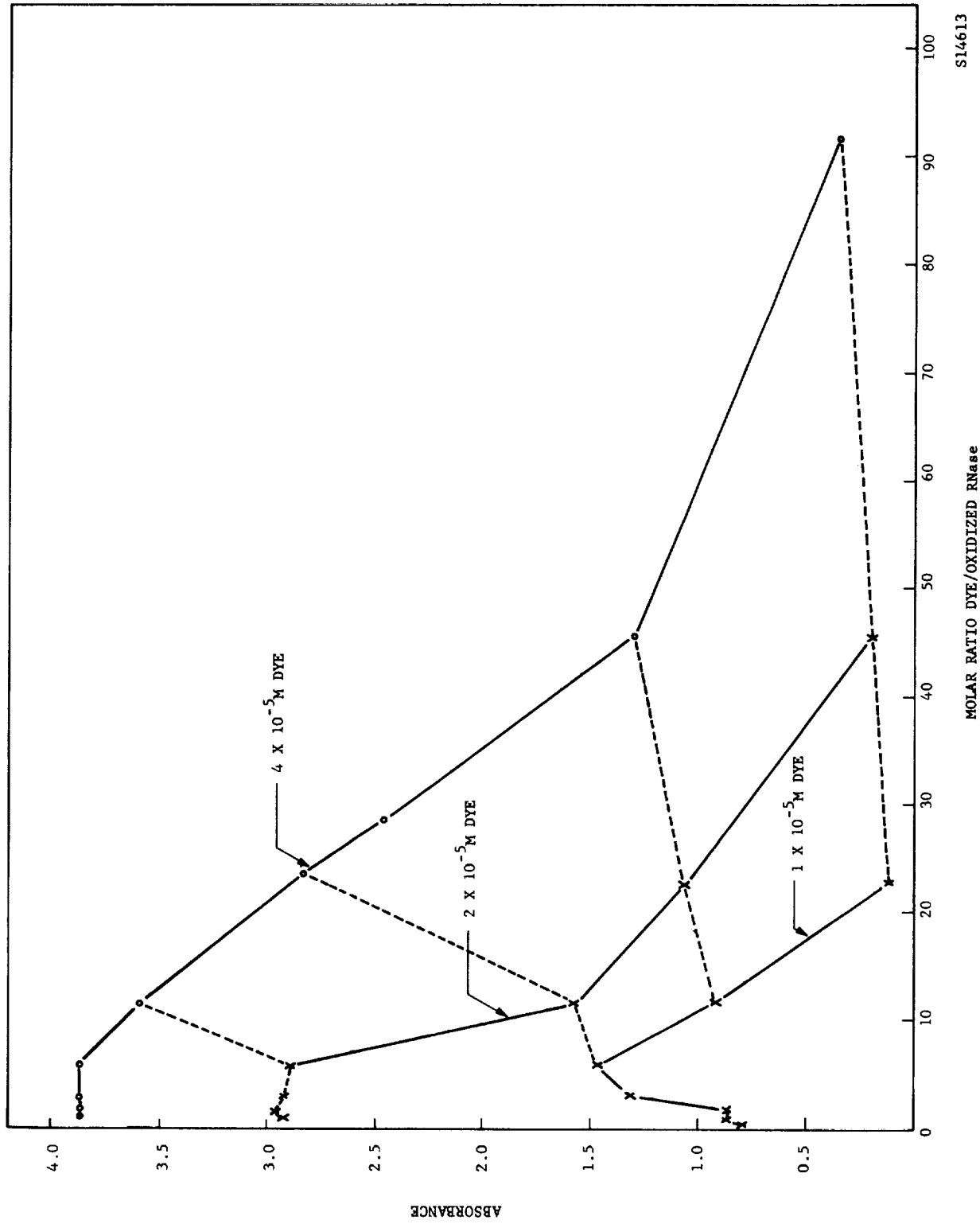


FIGURE 14. STOICHIOMETRY OF DYE-oxPNAse REACTION

The absorbance was measured at 650 mμ using 1.0 or 0.1 cm cells and water as the blank. All values are adjusted to a 1.0 cm path length. The dotted lines connect points of equal oxRNAse concentration.

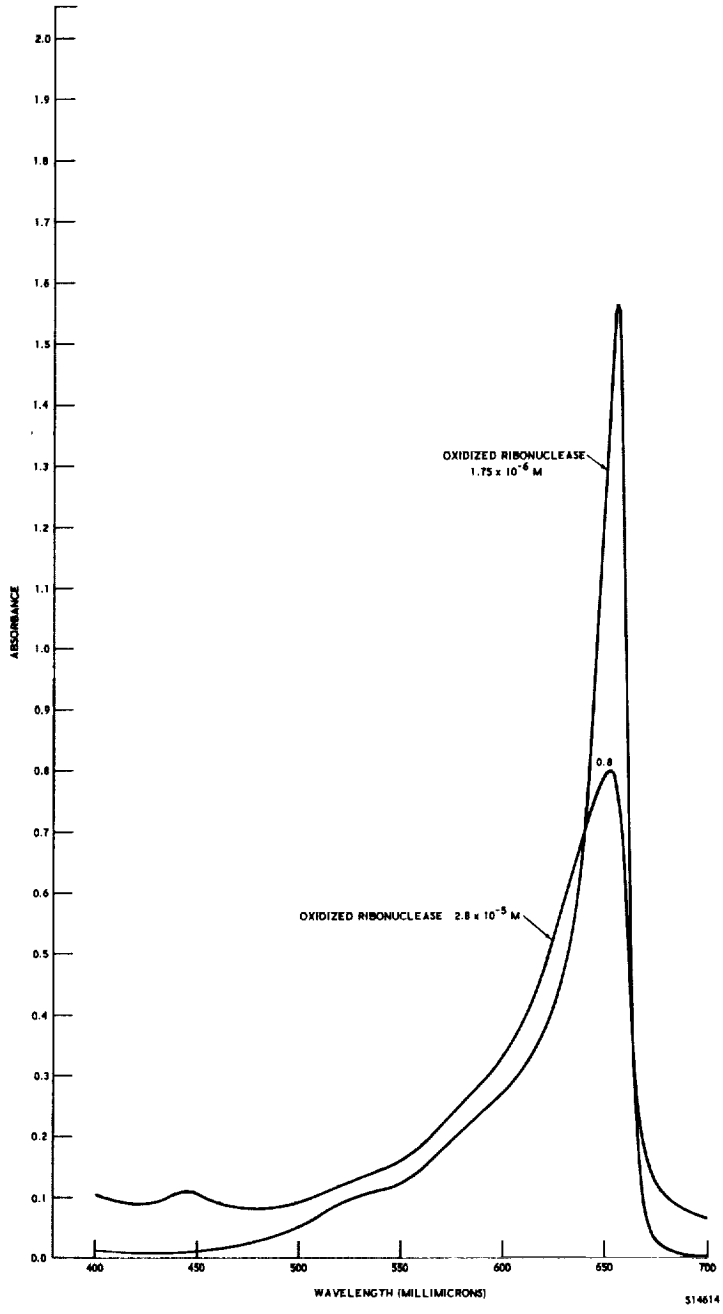


FIGURE 15. EFFECT OF PROTEIN-DYE RATIO ON THE ABSORPTION SPECTRUM OF THE OXIDIZED RIBONUCLEASE-DYE COMPLEX

The dye concentration was 1 x $10^{-5}$M and measurements were made in 1.0 cm cells against a water blank.

When the molar ratio of dye to oxRNAse is less than 5.7 there is insufficient dye to satisfy the stoichiometry of the dye-oxRNAse reaction, and this can cause alterations in the absorption spectrum of the oxRNAse-dye complex. In addition, high dye concentrations favor higher equilibrium concentrations of the dye-oxRNAse complex and therefore increase the sensitivity for detecting the dye-oxRNAse complex by spectrophotometric means.

It is also very interesting to note that when the molar ratio of dye to oxRNAse is 5.7 or less only the 650 mμ maximum, which represents a very large polymer, is formed. On the other hand, at molar ratios of dye to oxRNAse greater than 5.7, the 650 mμ maximum and several other maxima representing the monomeric and low polymeric forms, are seen (Table IX). This strongly suggests that the smaller polymers can form only after large polymer formation is complete. Therefore, the maxima representing the monomeric and low polymeric forms are not a result of the first stages of large polymer formation. They are instead due to interaction of the dye with the oxRNAse in a manner which is different from that leading to large polymer formation and probably reflect a fundamental property of the oxRNAse molecule.

For the DNA-dye complex the result was simply a linear increase in the intensity of the absorption peak until either the amount of dye or DNA became a limiting factor. The limiting molar ratio of dye to DNA phospate was 1:1 for either native or denatured DNA showing that no additional binding sites were produced by denaturation. At the limiting molar ratio no free dye was present in ultrafiltrates or in the supernate of a dye-DNA mixture subjected to ultracentrifugation. This indicates that the equilibrium is far on the side of complex formation.

## 3.5 EFFECT OF CHANGES IN MACROMOLECULE STRUCTURE ON THE ABSORPTION SPECTRUM OF THE MACROMOLECULE-DYE COMPLEX

### 3.5.1 DENATURATION OF DEOXYRIBONUCLEIC ACID (DNA)

Since the denaturation of DNA causes a transition from a rigid, two-stranded helix to a flexible, random coil, it might be expected that the nature of the dye-DNA complex will change with denaturation and a change in the absorption spectrum of the DNA-dye complex result. To test this idea, solutions of salmon sperm (Calif. Corp. for Biochem. Research) and calf thymus (Worthington Biochem. Corp.) DNA were prepared as in Section 3.3.4 and progressively denatured by heating or by altering the pH.

Both calf thymus DNA and salmon sperm DNA were denatured by heating solutions for 1 hour at various temperatures. The helix to coil

TABLE IX

MAXIMA FOUND AT DIFFERENT MOLAR RATIOS
OF DYE TO OXIDIZED RIBONUCLEASE

(Measurements were made in 1.0 cm cells against a water blank. The solutions were buffered at pH 7.0 with 0.017M cacodylic acid buffer.)

| Molar Ratio Dye/oxRNAse | Wave Lengths of Observed Absorption Maxima (mμ) |
|---|---|
| 0.71 | 650 |
| 1.42 | 650 |
| 2.85 | 650 |
| 5.70 | 650 |
| 11.4 | 650, 613, 576 |
| 14.2 | 650, 606, 530 |
| 38 | 650, 580, 530 |

transition was followed by the increase in ultra-violet absorption at 259 mμ. Aliquots of the DNA solutions were mixed with the aqueous dye solution and the absorption spectrum of the DNA-dye complex determined. In each case, the UV absorbance of the heated samples was compared to that of a sample kept at 25°C and the absorption spectrum of the heated DNA-dye mixture was compared to that of a DNA-dye solution prepared with DNA kept at 25°C.

In the samples heated to temperatures of less than 85°C virtually no change was found in the absorbance at 259 mμ or the absorption spectrum of the DNA-dye complex. The salmon sperm DNA formed a soluble complex with the dye in each case, but the calf thymus DNA-dye complex precipitated upon standing when DNA which had been heated to less than 87.5°C was added to the dye solution. Denaturation of DNA by heat caused a maximum increase of 28 percent in the UV absorbance of the calf thymus DNA solution and a maximum increase of 25 percent in the absorbance of the salmon sperm DNA. The melting point of both DNA samples appeared to be at about 85°C and when solutions heated to temperatures of 85°C or higher were mixed with the dye solution, the absorption spectrum was considerably different from the native DNA-dye complex. The peak at 570 mμ became a shoulder and a new peak appeared at 535 mμ. The intensity of the 535 mμ peak increased with the degree of denaturation, and the shape of the absorption spectrum of the completely denatured DNA-dye complex became the same as the RNA-dye complex (Figure 8). Thus it appears that the 570 mμ peak represents native DNA and the 535 mμ peak denatured DNA. Therefore, the ratio of the absorbance at 535 mμ to the absorbance at 570 mμ ($A_{535}/A_{570}$) should be a criterion of the degree of denaturation of DNA, and a large amount of denaturation would be indicated by a high ratio. Figure 16 is a plot of the percent of the total change in absorbance of the DNA solution at 259 mμ and of the percent total change in $A_{535}/A_{570}$ of the dye-DNA complex. This plot shows that the fractional changes in the $A_{535}/A_{570}$ follow the fractional changes in the ultra-violet absorption in most respects. However, for both salmon sperm and calf thymus DNA the $A_{535}/A_{570}$ curve is steeper than the curve for absorbance at 259 mμ and the UV absorption continues to increase with temperature up to 100°C whereas the change in $A_{535}/A_{570}$ is complete at 95°C.

Salmon sperm DNA was also denatured by additions of small amounts of 1N HCl or NaOH, and the effects of this denaturation on the UV absorption of the DNA and the absorbance of the dye-DNA complex at 535 and 570 mμ were determined. The order of the events did not appear to be critical since the same results were obtained when the pH of the DNA solution was adjusted and then the dye added or the DNA and dye mixed and the pH altered. The effects of pH on the $A_{535}/A_{570}$ ratio of the dye-DNA complex and the absorbance of the DNA solution at 259 mμ are shown in Figure 17. The maximum increase in absorbance of the DNA was 28.7 percent (pH 2.2) and in the absence of the

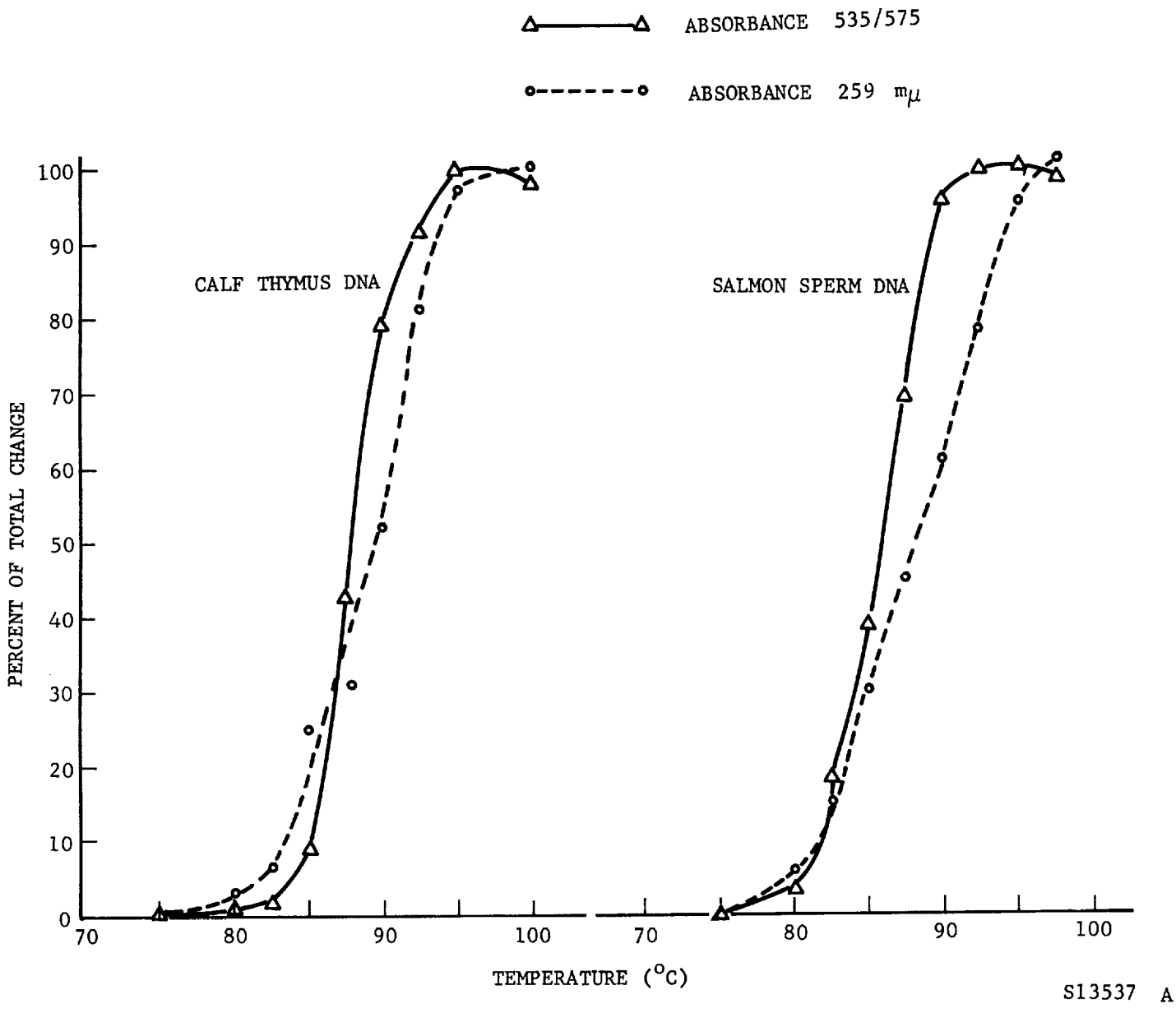


FIGURE 16. EFFECT OF HEAT ON THE ABSORBANCE OF DNA AT 259 mμ AND ON THE RATIO OF THE ABSORBANCE OF THE DYE-DNA COMPLEX AT 535 mμ TO THE ABSORBANCE AT 575 mμ (A535/A575).

Calf Thymus DNA and salmon sperm DNA were prepared as dilute solutions (about 0.009 percent) in cacodylic acid buffer containing 1.0 mM $MgCl_2$. Samples of each DNA solution were heated for 1 hour at the temperatures indicated in the figure and the absorbance at 259 mμ compared to a sample maintained at 25°C. Aliquots of the DNA solutions were then mixed with equal volumes of $8 \times 10^{-5}$M dye solution, the absorbance determined at 535 and 570 mμ and the ratio A535/A575 compared to the 25°C sample.

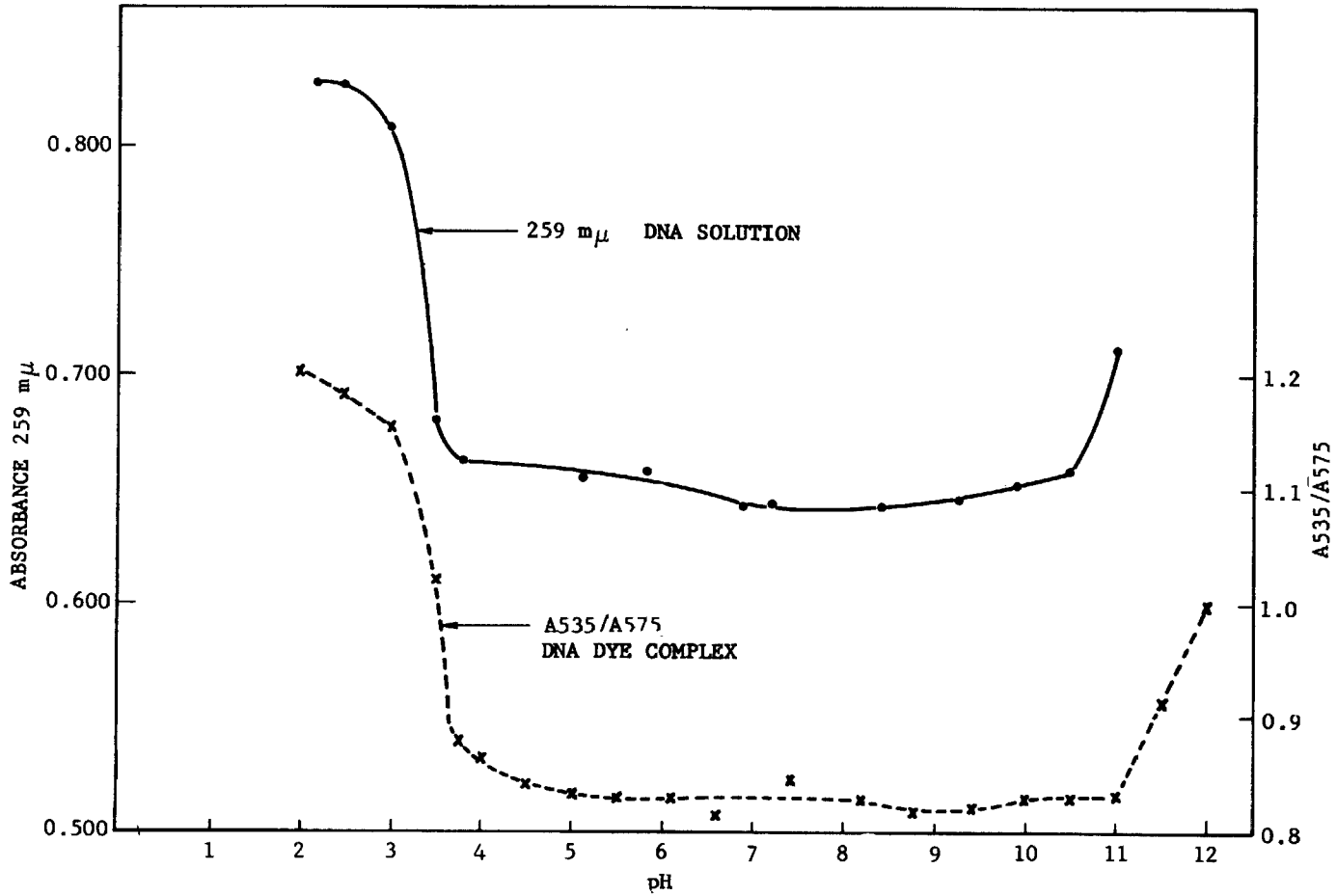


FIGURE 17. EFFECT OF pH ON THE ABSORBANCE OF DNA AT 259 mμ AND ON THE RATIO OF THE ABSORBANCE OF THE DYE-DNA COMPLEX AT 535 mμ TO THE ABSORBANCE AT 570 mμ (A535/A570). S13536 A

Salmon Sperm DNA was prepared as a 0.014 percent solution in 1.0 mM $MgCl_2$. The pH of one sample of the solution was decreased by the addition of 1 N HCl and the pH of a second sample was increased by the addition of 1 N NaOH. The absorbance of the solutions at 259 m was determined at the pH indicated in the figure. A solution containing DNA-DYE was prepared by mixing equal volumes of the DNA solution and 8 x $10^{-5}$M DYE (DNA 0.007 percent, DYE 4 x $10^{-5}$M) and the pH of two samples of the mixture were altered as before. At the pH indicated in the figure the absorbance of the solution was determined at 535 and 575 mμ.

dye the DNA precipitated at pH 11.5. The shapes of the curves obtained are very similar and the $A_{535}/A_{570}$ ratio reflects the degree of denaturation of the DNA as evidenced by the increase in UV absorbance.

### 3.5.2 OXIDATION OF RIBONUCLEASE (RNAse)

Native RNAse solutions containing 0.06 to 0.002 percent RNAse were prepared in cacodylic acid buffer from a crystalline sample of bovine pancreatic RNAse supplied by Mann Research Laboratories, Inc. These solutions were mixed with dye, and the absorption spectra of the mixtures determined and compared to the absorption spectrum of an aqueous dye solution. None of the native ribonuclease solutions had an effect upon the absorption spectrum of the dye*.

Pancreatic RNAse is a symmetrical and tightly-coiled molecule in solution[18] and the failure of the dye to interact with this protein may be related to this property. If this is the case, it is possible that disruption of the coiled structure could lead to a configurational change which will permit the protein and dye to interact. To test this idea, the secondary structure of the ribonuclease was altered by treatment with 8M urea or oxidation with performic acid. The denatured ribonuclease solutions were then mixed with the dye and the absorption spectra of the solutions determined. Treatment of the ribonuclease with 8M urea did not change its reaction with the dye. However, oxidation with performic acid produced a product which induced large changes in the absorption spectrum of the dye. A 0.002 percent solution of the oxidized ribonuclease caused the formation of intense maxima at 648 mμ and 575 mμ (Figure 18). Thus, when the disulfide bonds of the 1/2 cysteine residues are broken and cysteic acid residues formed, the secondary structure of ribonuclease is sufficiently altered to allow the dye to interact with this protein.

## 3.6 INTERACTION OF DYE WITH HETEROGENEOUS SUBSTANCES

### 3.6.1 MICROORGANISMS

The interaction of the dye with various microorganisms is of interest from the standpoint of the detection and possible characterization

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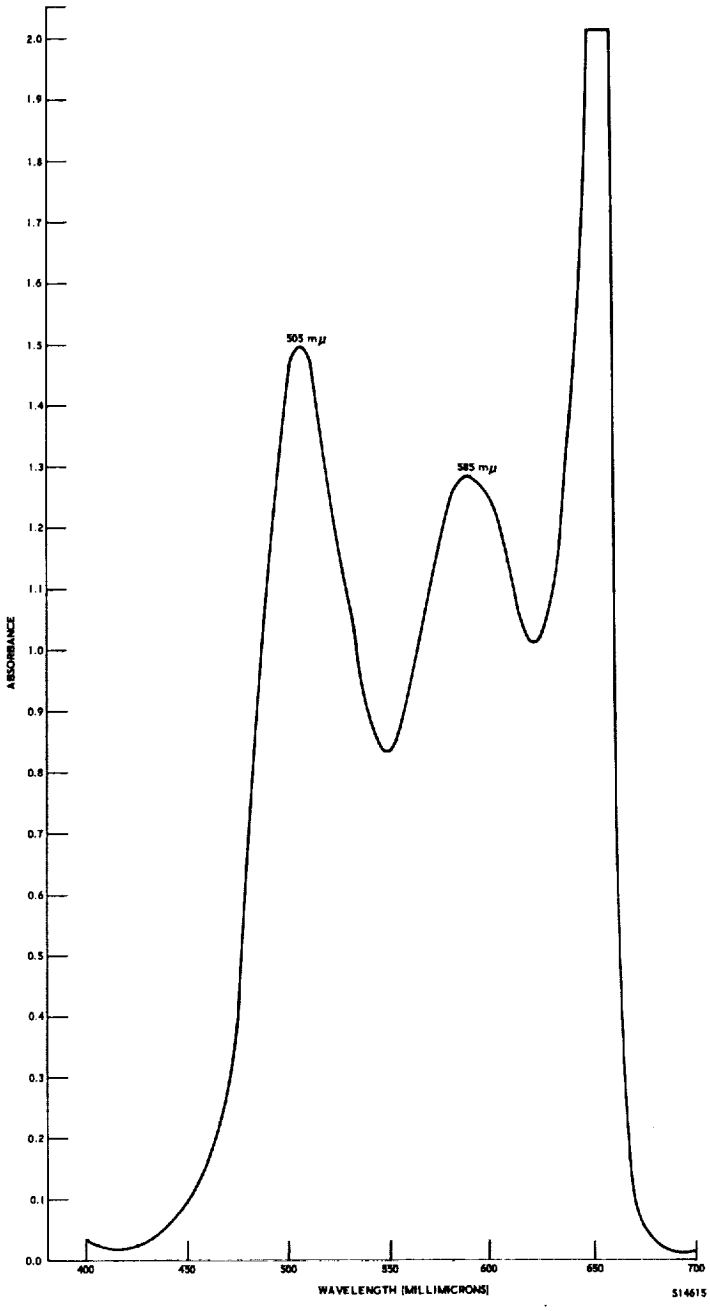
*It is interesting to note that a sample of a 0.02 percent solution of crystalline RNAse obtained from Calif. Corp. for Biochem. Research affected the absorption spectrum of the dye in much the same manner as 0.002 percent solution of Mann RNAse which had been oxidized with performic acid.




S14615 FIGURE 18. ABSORPTION SPECTRUM OF DYE PLUS OXIDIZED RIBONUCLEASE

The solution contained 0.002 percent oxidized ribonuclease, 4 x $10^{-5}$M dye and was buffered at pH 7.0 with 0.017M cacodylic acid buffer. Measurements were made in 1.0 cm cells using water as a blank.

of these life forms. Solutions containing trace amounts of microorganisms were prepared from fresh cultures. These solutions were mixed with the dye solution and buffered at pH 7.0 with 0.017M cacodylic acid buffer. The absorption spectra of the mixtures were determined and the wave lengths at which new maxima appeared were noted. The results are shown in Table X. The data show that very small amounts of the organisms were effective in causing large changes in the absorption spectrum of the dye. In addition, it is especially noteworthy that no two organisms produced changes which were exactly identical and, in fact, the changes observed were often quite dissimilar (Figure 19). It therefore appears that the reaction of the dye with microorganisms can serve as a basis for their detection and possibly as a means for characterizing these life forms.

### 3.6.2 SOIL EXTRACTS

The ability to detect trace amounts of biological macomolecules in heterogeneous samples such as soil is a very important consideration for the application of the dye reaction to the detection of extraterrestrial life. The information obtained in this study suggests that the detection of macromolecules in soil may be accomplished rapidly by simply observing the reaction of the dye with aqueous extracts obtained from relatively small amounts of soil. Therefore, an experiment was designed to examine this possibility.

A sample of a sandy, relatively nonfertile soil was sifted through a fine screen and thoroughly mixed to obtain a uniform preparation. This preparation was designated as "native soil". The organic matter contained in a sample of this native soil was removed by heating it to a high temperature and the heated sample was designated as "incinerated soil". Zinc sulfate or $\beta$-lactoglobulin was added to 1 g samples of both native and incinerated soil and to 10 ml of distilled water. These soil samples and 1 g samples of native and incinerated soil, to which zinc sulfate or $\beta$-lactoglobulin had not been added, were mixed with 10 ml of distilled water and the solids were allowed to settle out by standing. Aliquots (2 ml) of the zinc sulfate and $\beta$-lactoglobulin solutions and the supernates from the soil samples were then mixed with 2 ml of $8 \times 10^{-5}$M dye. Other aliquots of the soil extracts and the zinc and $\beta$-lactoglobulin solutions were dialyzed against water and 2 ml samples of the dialyzed samples were mixed with 2 ml of $8 \times 10^{-5}$M dye. The absorption spectrum of each of the mixtures was determined and the wavelength of any new absorption maxima noted. The results are presented in Table XI.

The data show that the supernate of the native soil sample reacted with the dye to form new absorption maxima, whereas the extract from the incinerated soil did not change the absorption spectrum of the dye. This indicates that the changes observed were due to substances which were

TABLE X

NEW ABSORPTION MAXIMA FORMED BY DYE IN THE PRESENCE OF MICROORGANISMS

(The dye concentration was $4 \times 10^{-5}$M and the mixtures were buffered at pH 7.0 with 0.017M cacodylic acid buffer. Measurements were made in a 1.0 cm cell using water as the blank.)

| Microorganism | Concentration of Microorganism (percent based on wet weight) | Wave Length of New Absorption Maxima (mμ) |
|---|---|---|
| Escherichia coli | 0.00125 | 645 |
| Bacillus subtilis | 0.0016 | 645,580 |
| Clostridium kluyveri | 0.0067 | 650,570,535,450 |
| Rhodotorula | 0.004 | 650,580 |
| Aspergillus niger | 0.002 | 640,570 |
| Chlorella | 0.0086 | 535,450 |
| Tobacco Mosaic Virus | 0.002 | 570,468 |
| Pollen(Bird of Paradise) | 0.005 | 650,570 |

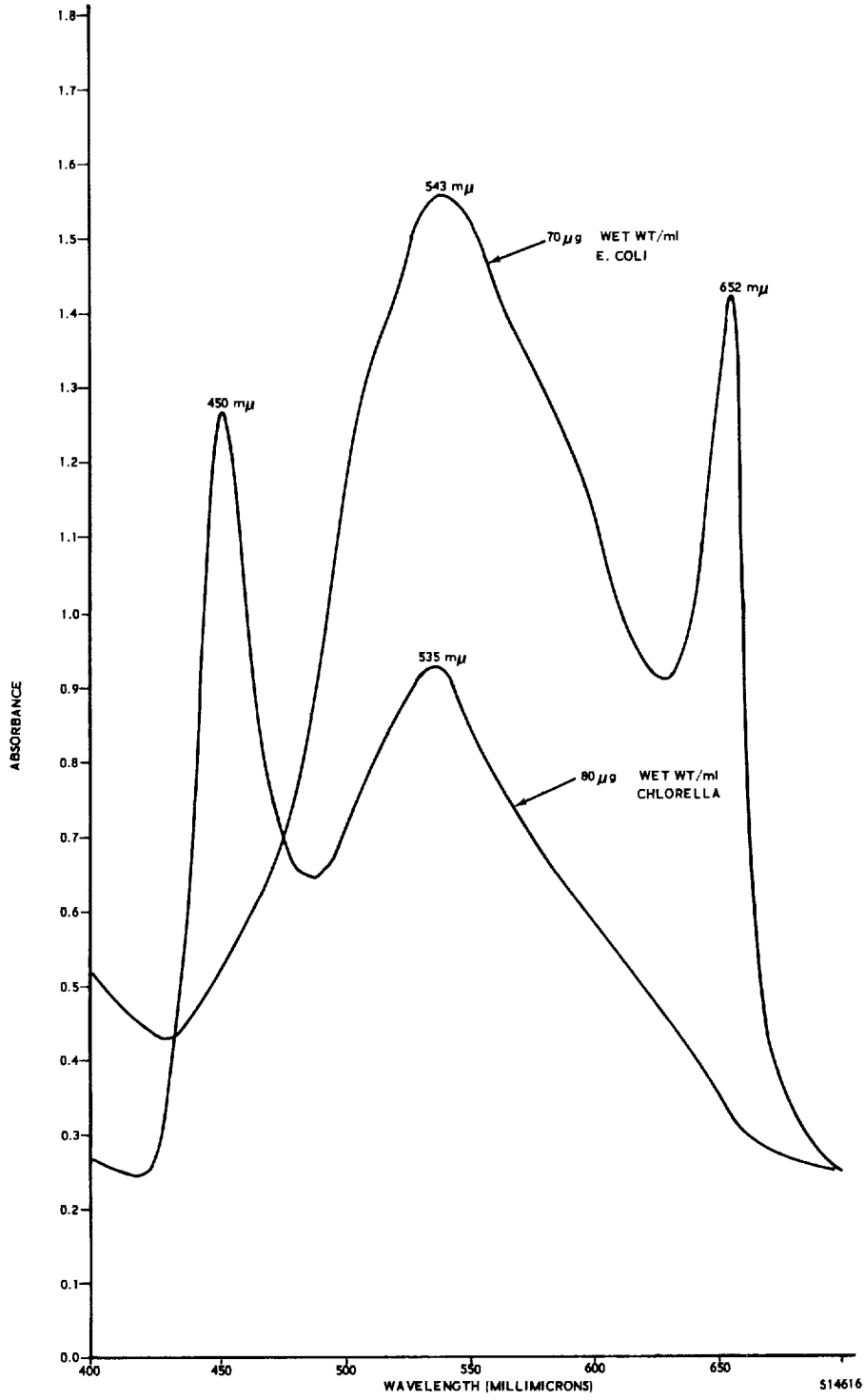



FIGURE 19. ABSORPTION SPECTRA OF DYE PLUS MICROORGANISMS

The dye concentration was 4 x $10^{-5}$M and the solutions were buffered at pH 7.0 with 0.017M cacodylic acid. Measurements were made in 1.0 cm cells using water as a blank.

TABLE XI

INTERACTION OF THE DYE WITH SOIL EXTRACTS

| Preparation | Pretreatment | Wave Lengths of New Absorption Maxima (mμ) |
|---|---|---|
| Extract of native soil | None | 650, 532 |
| | Dialysis | 650, 530 |
| Extract of incinerated soil | None | None |
| β-lactoglobulin solution (0.005 percent) | None | 640, 570 |
| | Dialysis | 640, 570 |
| Extract of β-lactoglobulin (0.005 percent) plus incinerated soil | None | 640, 570 |
| | Dialysis | 640, 570 |
| Zinc sulfate solution (0.02M) | None | 650, 575 |
| | Dialysis | None |
| Extract of zinc sulfate (0.02M) plus incinerated soil | Dialysis | None |

heat labile. Dialysis of the $\beta$-lactoglobulin solution, $\beta$-lactoglobulin plus incinerated soil or the supernate from the native soil did not alter the reaction of these extracts with the dye. On the other hand, when the zinc sulfate solution or the zinc sulfate plus incinerated soil extract were dialyzed they no longer caused the formation of new absorption maxima. Therefore, the dialysis was sufficient to remove small molecules, but it did not remove macromolecules or alter their reaction with the dye. These results indicate that, for the soil tested, the amounts of salts or other reactive small molecules present in the extract were insufficient to cause changes in the dye spectrum, whereas there were enough heat labile, non-dialyzable substances present to cause marked changes in the absorption spectrum of the dye (Figure 20). This strongly suggests that organic macromolecules were present in the extract of the native soil sample and that the reaction of the dye with the crude soil extract can serve as a method for their detection.

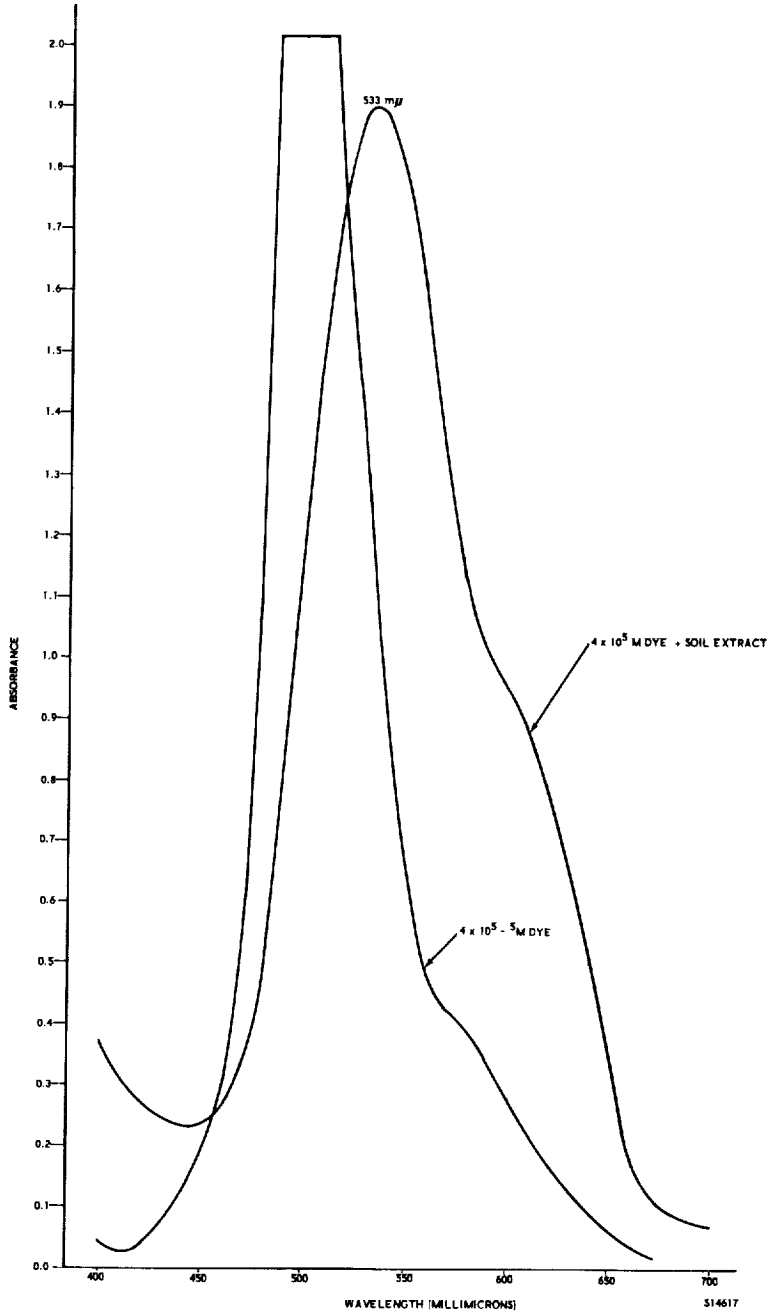



FIGURE 20. ABSORPTION SPECTRUM OF AQUEOUS NATIVE SOIL EXTRACT PLUS DYE

The mixture was composed of 2 ml of 8 x $10^{-5}$M dye plus 2 ml of a soil extract obtained from a mixture of 1 g of soil with 10 ml of distilled water. Measurements were made in 1.0 cm cells using water as a blank.

# SECTION 4

# DISCUSSION

It has been shown that proper manipulation of the temperature and solvent composition will cause the cationic dye 3,3'diethyl-9-methyl 4,5,4'5' dibenzothiacarbocyanine bromide to form a number of aggregated species in solution. This phenomenon is not unique, since many other dyes are known to exhibit this property. However, the fact that at least six different maxima can be demonstrated is noteworthy. These maxima, which appear to represent species in different states of aggregation, may be arranged in order of increasing degree of dye aggregation as follows: 570, 555, 535, 510, 450 and 650 mμ. The 570 mμ peak represents the monomer and the 650 mμ peak a very large polymer. The other maxima presumably are characteristic of species which have aggregation states between these extremes. Over the concentration range which has been employed in this study, the dye exists in aqueous solution as the species having an absorption maximum at 510 mμ (e.g., in a state of intermediate aggregation). Thus it is possible for the dye normally present in these aqueous solutions to either increase or decrease its state of aggregation, and to do so in several discrete steps. Therefore, reactions of the dye with macromolecules in aqueous solution can result in the formation of new absorption bands due to: (a) further polymerization of the dye, (b) depolymerization of the dye, or (c) a combination of "a" and "b".

However, in order for any of these events to occur, it is necessary for the macromolecule to contain a large number of accessible, ionizable, anionic groups. Since the dye is cationic, this indicates that electrostatic forces play an important role in the dye-macromolecule reaction. The arrangement and nature of the anionic sites also appear to be important factors in determining the aggregation state of the adsorbed dye. Although detailed information on the factors involved must await further investigations, one general feature is apparent: macromolecules whose binding sites are composed of a number of different anionic groups which are free to assume a

number of positions (e.g., oxidized ribonuclease and β-lactoglobulin) interact with the dye to form many different absorption maxima. Macromolecules whose binding sites are composed of only one type of regularly spaced anionic site (e.g., DNA, RNA, poly-L-aspartic acid and hyaluronic acid) interact with the dye to form a single absorption band, which may represent either depolymerized or highly polymerized dye molecules.

The interaction of the dye with DNA is of special interest. Native DNA binds the dye as a monomeric species (single absorption peak at 575 mμ); as estimated from the limiting molar ratio of dye to DNA phosphate, each DNA phosphate group binds one dye molecule. This complex is very stable, since neither changes in pH nor large increases in temperature cause release of the dye. However, when the DNA is denatured, either prior to mixing with the dye or after the dye-DNA complex has been formed, the maximum at 575 mμ is replaced in direct proportion to the extend of denaturation by a new maximum at 535 mμ, indicating that an increase in polymerization of the dye has occurred. Thus, it can be concluded that the dye shows no tendency to aggregate when bound to native DNA, but that it polymerizes as the DNA is denatured. It is interesting to speculate on the nature of this polymerization. It has been shown that the number of dye molecules bonded per phosphate group is the same in native or denatured DNA and that the complex is very stable. In addition, the effect of denaturation is the same whether the DNA is denatured before or after it is bound to the dye. Thus, it appears that the dye remains bound to the DNA during the denaturation process and that the binding sites of the denatured and native DNA are the same and of equal number. However, the dye binding sites of the rigid two-stranded native DNA molecule are severely constrained, whereas those of the random coiled, denatured DNA are not. Thus, in the case of denatured DNA, it is possible for the dye molecules bound to the anionic sites to interact with each other. This interaction can alter the energy levels of either the ground or excited states of the dye, or both, causing a spectral shift in the dye to shorter wave lengths typical of the more aggregated state. The extent of the interactions of the bound dye molecules would therefore indicate the randomness of the DNA molecule and its degree of denaturation. If this hypothesis is correct, the interactions of the large number of adsorbed dye molecules should make the average conformation of the denatured DNA-dye complex quite different from that of denatured DNA.

Unlike the complex formed by the reaction of acridine orange with DNA[3,4,19], the thiacarbocyanine dye-DNA complex is not easily dissociated by changes in ionic strength, pH or temperature. This suggests that the changes in the absorption spectrum of the dye which take place on denaturation of the DNA may be more useful as a criterion of the extent of denaturation than those found with acridine orange.

The detection of biological macromolecules by their reaction with the dye appears to be quite feasible. Alterations in the absorption spectrum of the dye take place in the presence of trace amounts of these substances and the methodology is extremely simple. The simplicity of the method recommends it for systems which must function by automated procedures. Therefore, it appears to be especially suited as a tool for the detection of life on other planets. The detection and study of extraterrestrial life in the universe is considered by many scientists to be the most important objective in space biology[20]. It appears that in the immediate future the exploration of space will be largely confined to our own solar system[21] and it is generally concluded that within our solar system, other than Earth, the planets Mars and Venus offer the best possibilities of supporting life[22-24]. This conclusion stems from the belief that life, if it exists on these planets, must have arisen in much the same manner as on Earth. If life can begin only in the same manner as on Earth, it is reasonable to expect that the same chemical constituents as those on Earth would be involved in the creation of extraterrestrial life, at least in the initial stages. On Earth, the presence of proteins, polynucleotides and polysaccharides in the environment appears to be the unique result of the action of living organisms and it is thought that the primeval form of life was protein-centered, just as all current terrestrial life is[25,26]. Therefore, it is reasonable to base the detection of Martian and Venutian life on the presence of macromolecules which have the characteristics of proteins, polynucleotides or polysaccharides. From the results of the experiments presented in this investigation it appears that the interaction of the dye 3,3'diethyl-9-methyl 4,5,4'5' dibenzothiacarbocyanine bromide with proteins, polynucleotides and substituted polysaccharides can form the basis for the detection of a vast number of these substances in trace amounts.

The effects of environmental variables on the dye have been studied and there appear to be no factors which will seriously handicap its use in a detection system. The deleterious effects of high temperatures, exposure to high light intensity and large changes in pH can be eliminated. Therefore, the sensitivity, simplicity and reliability of the system are the most important factors to be considered.

In a system in which realistic changes in the absorption spectrum are observed, the minimum detectable macromolecule concentration is of the order of 0.0005 to 0.00005 percent, depending upon the macromolecule tested, and therefore the sensitivity appears to be adequate.

The simplicity of the system is one of its outstanding characteristics, and in essence it is only required that the macromolecule come in contact with the dye in an aqueous environment. For most macromolecules, the optimum conditions for dye-macromolecule interaction appear to include a pH of 7-9, a dye concentration of $4 \times 10^{-5}$M and a reaction temperature between 20 and 35°C. All of these considerations can be easily provided.

In order for the system to be a reliable indicator for the presence of proteins, polynucleotides and polysaccharides, it should respond to a variety of these compounds in a characteristic way, but not to other types of substances. Of the substances tested, only proteins, polynucleotides and substituted polysaccharides reacted with the dye to cause new absorption maxima when present in trace amounts. However, at increased concentrations, inorganic ions (particularly divalent ions), di- and tripeptides, and nucleotides also induced similar alterations in the absorption spectrum of the dye. The di- and tripeptides must be present in such large quantities before they cause changes that their reaction with the dye under realistic conditions is of little concern. On the other hand, small amounts of either inorganic salts or nucleotides cause alterations in the absorption spectrum of the dye. The induction of new absorption maxima by inorganic salts appears to be a much more serious problem than the reaction with nucleotides. Inorganic salts may occur in large amounts on extraterrestrial bodies and they are not indicative of life forms. Nucleotides will probably not exist in high concentrations; in any case, their detection would not be especially undesirable, since it is difficult to see how they could arise in large concentration without the presence of life.

In regard to differentiating between changes in the absorption spectrum of the dye caused by inorganic salts and macromolecules, the thermal stabilities of the dye aggregates induced by inorganic salts and nucleotides and the dye complexes formed with protein and polynucleotides are of interest. The stability of dye aggregates induced by inorganic salts and nucleotides are favored by low temperatures (7°C), and even with moderate increase in temperatures (31°C) these aggregates are dispersed. On the other hand, aggregation of the dye on proteins is favored by higher temperatures (40°C), and the stability of the dye-DNA complex does not appear to be significantly altered by temperature changes over the range 7 to 89°C. These differences in thermal stability may present an exceptionally simple method for distinguishing among changes in the absorption spectrum of the dye due to proteins, polynucleotides, and inorganic salts. It is possible that by simply determining the absorbance of the preparation at two temperatures, for example, 10 and 30°C, an immediate distinction can be made among these classes of compounds. An increase in temperature should decrease the response due to inorganic salts, accentuate the response caused by proteins, and not affect changes induced by polynucleotides.

Our studies with soil samples indicate that inorganic salts may not be important factors in the detection of macromolecules in soil. High molecular weight organic molecules were easily detected in soil extracts, prepared by simply mixing buffer solution and dry soil, and control experiments showed that the concentration of inorganic salts and other small

molecules present in the soil extract was not sufficient to give a false-positive reaction. However, these studies did not rule out the possibility that the reaction was due to a high molecular weight polyphosphate.

On the whole, the results obtained appear to be especially encouraging for the possible application of the dye reaction to the detection of trace amounts of macromolecules in heterogeneous samples such as would be examined on an extraterrestrial body. The reactions show high sensitivities, and they exhibit special characteristics which may yield considerable information about the nature of the macromolecules. The method appears to be suitable for the detection of a large number of proteins and, in addition, the dye exhibits rather specific and sensitive reactions with nucleic acids and long-chain polysaccharides. It is also of considerable interest that some rather detailed information about macromolecules (e.g., the degree of DNA denaturation) can be deduced by examination of the absorption spectrum of the dye-macromolecule complex.

# SECTION 5

## SUMMARY

(1) A method of detecting proteins and other macromolecules in trace amounts, based upon the observation of spectral changes due to aggregation of a carbocyanine dye when it is adsorbed on the macromolecular structure, was investigated.

(2) Of eight dyes tested, 3,3'diethyl-9-methyl 4,5,4'5' dibenzothiacarbocyanine bromide was shown to react most favorably with macromolecules to provide the information desired.

(3) The effects of pH, temperature, and electromagnetic radiation on the stability of the dye were determined. The dye was stable over the pH range 3.8 to 9.6 and unaffected by storage at temperatures below 60°C, but it was unstable when exposed to intense light. The use of brown glass or aluminum covered glassware prevented the photo-destruction of the dye without hindering the experimental procedures.

(4) The effects of pH, solvent, dye concentration, temperature, and inorganic ions on the wave length of the dye absorption maximum were ascertained. The pH had no effect on the position of the absorption maximum, but proper manipulation of the other variables produced changes in the wave length of the maximum. The information obtained from these investigations was used to determine the maxima which are indicative of different states of dye aggregation. These maxima were found at 575, 555, 535, 510, 450 and 650 mμ(J-band) and represent increasing degrees of aggregation in the order given.

(5) The interactions of the dye with inorganic salts, microorganisms, pollen, polypeptides, simple proteins, conjugated proteins, synthetic polypeptides, polynucleotides, carbohydrates, amino acids,

pyrimidine and purine bases, nucleosides and nucleotides were all investigated. In trace amounts (less than 0.002 percent) only proteins, synthetic polypeptides, nucleic acids, microorganisms, pollen and substituted polysaccharides caused changes in the absorption spectrum of the dye. Mono-, di- and trisaccharides, purine and pyrimidine bases, amino acids and nucleosides had no effect. Polypeptides and nucleotides were usually effective only at higher concentrations and the action of the inorganic salts was found to depend upon the nature of the anion. Divalent anions were very effective, and small amounts induced the formation of J-bands. On the other hand, monovalent anions were much less effective, and relatively large amounts were required to induce the formation of a J-band.

(6) Studies were conducted to determine the influence of the pH, temperature, and dye-macromolecule ratio on the spectral changes induced by macromolecules. Alterations in pH affected each protein-dye complex somewhat differently. As judged by decreases in absorbance at the characteristic maximum, adjustment of the pH to 3 or less prevents aggregation of the dye on proteins, whereas a pH of 7 to 9 is conducive to interaction of the dye with protein. On the other hand, interaction of the dye with DNA was virtually unaffected by pH changes, but important alterations in the wave length of the absorption maximum occurred due to denaturation of the DNA. The influence of temperature on the dye-macromolecule complex and the dye aggregation formed in the presence of inorganic salts was especially significant. High states of dye aggregation by inorganic salts was favored by temperatures of less than 20°C, and at higher temperatures the aggregation was dispersed. On the other hand, the aggregation of the dye when adsorbed on a protein was favored by higher temperatures and the interaction of the dye with DNA was not significantly altered, but the position of the maximum was changed when the DNA was denatured. The stoichiometry of the dye macromolecule reaction was investigated in the case of DNA and oxidized ribonuclease. For the DNA-dye reaction the result was mainly a linear increase in the intensity of the peak until either the amount of dye or DNA became a limiting factor. The limiting ratio of dye to phosphate groups was 1:1 for both native and denatured DNA. For oxidized ribonuclease (oxRNAse), the absorbance of the dye-protein complex at 650 mμ increased with either increasing oxRNAse or dye concentration until a limiting ratio of 5.7:1 dye molecules to protein molecules was reached. When the ratio was greater than 5.7, new peaks formed at shorter wave lengths.

(7) The effect of changes in the macromolecule structure on the absorption spectrum of the macromolecule-dye complex was investigated by comparing the absorption spectrum of native DNA-dye complex to the spectrum of denatured DNA-dye complex, and the reaction of the dye with native ribonuclease to its reaction with oxidized ribonuclease. The interaction of the dye with denatured and native DNA produced highly

interesting and significant results. Completely denatured DNA reacted with the dye to produce an absorption peak at 535 mμ, whereas native DNA induced an absorption peak at 575 mμ. It was found that the ratio of the absorbance at 535 mμ to the absorbance at 570 mμ could serve as an accurate and sensitive measure of the degree of denaturation. Native ribonuclease solutions did not interact with the dye to produce new absorption peaks. On the other hand, oxidized ribonuclease reacted with the dye to form a number of new maxima. Thus, the reaction of the dye with ribonuclease can give information about the state of the disulfide bridges in this molecule.

(8) The reaction of the dye with samples of a relatively non-fertile, sandy soil was investigated. Large increases in absorbance in the 640 mμ region of the spectrum were observed, and an intense new absorption peak was formed at 535 mμ. Control experiments indicated that these changes in the absorption spectrum were due to heat labile macromolecules.

(9) It was concluded that the results are especially encouraging for the possible application of the dye reaction to the detection of trace amounts of macromolecules in heterogeneous samples which might be analyzed on extraterrestrial bodies in the search for evidence of life.

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